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(71) Applicant: THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US).

(72) Inventor: REEVES, Steven, A.; 18 Davis Avenue, Arlington, MA 02174 (US).

(74) Agent: FRASER, Janis, K.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).

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(54) Title: CONTROLLED GENE PRODUCT DELIVERY FROM A REGULATABLE RETROVIRAL VECTOR

(57) Abstract

An inducible or regulatable retroviral vector for the controlled delivery of a gene product, and in particular, a tetracycline regulated retroviral vector for the controlled delivery of a gene product is disclosed. This retroviral vector contains the following DNA sequences operatively linked from 5' to 3': (a) DNA comprising a first promoter; (b) DNA comprising a second promoter different from the first promoter in (a) and different from the third promoter in (e); (c) DNA encoding a tetracycline regulator unit (tTA) under the transcriptional control of said second promoter; (d) DNA encoding a tetracycline response unit (tetO) in an antisense orientation relative to said DNA encoding said regulator unit of (c); and (e) DNA comprising a third promoter different from the first promoter in (a) and different from the second promoter in (b) wherein said DNA encoding said response unit of (d) is under the transcriptional control of said third promoter.

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CONTROLLED GENE PRODUCT DELIVERY FROM A
REGULATABLE RETROVIRAL VECTOR

Background of the Invention

10   This invention relates to an inducible or
regulatable retroviral vector for the controlled delivery
of a gene product and in particular to a tetracycline
regulated retroviral vector for the controlled delivery
of a gene product.

15   The introduction of heterologous genes into
cultured mammalian cells or tissues is fundamental for
understanding the biochemistry, genetics and function(s)
of genes and gene products. Much of the understanding of
biological processes has been determined by transfection,
20 electroporation, or viral delivery of genes into cultured
cells or tissues. More recently, gene therapy approaches
have emphasized the need for gene delivery vectors that
can efficiently introduce, and control the expression of
heterologous genes in mammalian cells.

25   An important feature of any gene delivery system
is the ability to regulate the expression of the
delivered gene. This is important in situations where
the translation product of the delivered gene is being
examined for its functional role in cell biology and in
30 therapeutic situations where the gene product is toxic or
must be maintained at appropriate levels. Ideally,
regulation of expression of the delivered gene should be
in an ON or OFF manner. Where when turned ON, gene
expression should be induced and when turned OFF gene
35 expression should be silenced.

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The tetracycline-controlled transactivator responsive promoter (Tet system) is a prokaryotic inducible promoter system which has been adapted for use in mammalian cells (Gossen, M. & Bujard, H. (1992) *Proc.*
5 *Natl. Acad. Sci USA*, 89, 5547-5551; Gossen, M., Bonin, A. L. & Bujard, H. (1993) *TIBS* 18, 471-475). In the previously developed Tet system, there are two components, each of which is carried on a separate plasmid. One component, the "response unit", is composed
10 of the *E. coli*-derived tetracycline resistance operon regulatory elements (*tetO*) embedded within a minimal CMV promoter. The second component, the "regulator unit", encodes a transactivator hybrid protein (tTA) composed of the tetracycline repressor (*tetR*) fused to the herpes
15 simplex virus (HSV) transactivator protein, VP16. Expression of a gene inserted downstream of the *tetO*/minimal CMV promoter in the response unit is highly dependent on tTA which binds *tetO* sequences through its *tetR* domain and recruits positively acting cellular
20 transcription factors through its VP16 domain.

Gene expression is inhibited by the addition of tetracycline, which binds the transactivator protein, causing it to dissociate from the *tetO*/minimal CMV promoter and leading to cessation of gene transcription.
25   Although the Tet system has proven to be an important tool for examining the effects of genes on cells, it is limited in its applications for the following reasons: 1)   For the Tet system to regulate the expression of a heterologous gene, two separate
30 plasmids must be introduced into the cells of interest. The introduction of the two plasmids into cells is performed using chemical or electrochemical procedures that are inherently inefficient in terms of the number of cells that take up the plasmid DNAs. The limitations of
35 these DNA transfection procedures restrict the use of the

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two plasmid-bas d Tet system to cultured cells and can
not be effectively used *in vivo* except in the case of
transgenic animals (Furth, P. A., Onge, L. S., Böger, H.,
Gruss, P., Gossen, M., Kistner, A., Bujard, H &
5 Hennighausen, L. (1994) *Proc. Natl. Acad. Sci. USA.* 91,
9302-9306; Fishman, G. I., Kaplan, M. L. & Buttrick, P.
M. (1994) *J. Clin. Invest.* 93 1864-1868);
           2)   The original two plasmid-based Tet system
(Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci.*
10 *USA*, 89, 5547-5551) still shows gene expression when the
system is switched OFF (W. P., unpublished data;
Ackland-Berglund, C. E. & Leib, D. A. (1995)
BioTechniques 18, 196-200). This means that the Tet
system allows low level uninduced gene expression, which
15 can be a significant problem in some applications.

Summary of the Invention

           In general, the invention features an inducible or
regulatable retroviral vector for the controlled delivery
of a gene product. In a preferred embodiments, this
20 vector is a tetracycline regulated retroviral vector.

           In an related aspect, this invention features a
tetracycline regulated retroviral vector capable of
delivering and controlling the expression of a
heterologous gene in mammalian cells, including the
25 following DNA sequences operatively linked from 5' to 3':
(a) DNA comprising a first promoter; (b) DNA comprising a
second promoter different from the first promoter in (a)
and different from the third promoter in (e); (c) DNA
encoding a tetracycline regulator unit (tTA) under the
30 transcriptional control of said second promoter; (d) DNA
encoding a tetracycline response unit (tetO) in an
antisense orientation relative to said DNA encoding said
regulator unit of (c); and (e) DNA comprising a third
promoter different from the first promoter in (a) and

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different from the second promoter in (b) wherein said
DNA encoding said r sponse unit of (d) is under the
transcriptional control of said third promoter.

In preferred embodiments, the first promoter (a)
5 in the above vector is specific for the cell type or for
tissue type that the vector infects. In various
preferred embodiments, this first promoter of (a) is
selected from the group consisting of 5' retroviral long
terminal repeat promoter, Rous Sarcoma Virus (RSV), Human
10 Immunodeficiency Virus (HIV), and phosphoglycerate
kinase.

Other preferred embodiments of this vector
includes DNA encoding a mammalian protein under the
transcriptional control of said first promoter (a).
15 Preferably, such proteins are selected from the group
consisting of ICE, TH, GDNF, and TH. In additional
preferred embodiments, this mammalian protein is
heterologous to the cells that the vector infects.

In various preferred embodiments, the addition of
20 tetracycline either increases (enhances) or inhibits
(suppresses) the expression of the mammalian gene.

In preferred embodiments, the second promoter (b)
of this vector is selected from the group consisting of
SV40 promoter, JC virus promoter, glial fibrillary,
25 nestin, PO, estrogen receptor, and phosphoglycerate
kinase. In other preferred embodiments, the third
promoter (e) in this vector is CMV promoter or thymidine
kinase.

In preferred embodiments, this vector further
30 includes an endonuclease site located in an antisense
orientation relative to the DNA encoding the regulator
unit. Preferably, this endonuclease site is Xho1, Spe1,
Sfi1, or Bstx1.

In preferred embodiments, this vector further
35 includes DNA encoding a selectable marker which is under

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the transcriptional control of the first promoter.
Preferably, the selectable marker is puromycin, neomycin,
hygromycin or thymidine kinase.

In another related aspect, this invention
5   features, a tetracycline regulated retroviral vector
capable of delivering and controlling the expression of a
heterologous gene in mammalian cells, including the
following DNA sequences operatively linked from 5' to 3':
(a) DNA comprising a first promoter; (b) DNA encoding a
10 selectable marker under the transcriptional control of
said first promoter in (a); (c) DNA comprising a second
promoter different from the first promoter in (a) and
different from the third promoter in (g); (d) DNA
encoding a tetracycline regulator unit (tTA) under the
15 transcriptional control of the second promoter; (e) an
endonuclease site located in an antisense orientation
relative to the DNA encoding said regulator unit of (d);
(f) DNA encoding a tetracycline response unit (tetO) in
an antisense orientation relative to the DNA encoding
20 said regulator unit of (d); and (g) DNA comprising a
third promoter different from first promoter in (a) and
different from second promoter in (c) wherein the DNA
encoding said response unit of (f) is under the
transcriptional control of the third promoter.

25   In preferred embodiments, the first promoter of
this vector is specific for the cell type or tissue type
that the vector infects. Preferably, this first promoter
is the 5' retroviral long terminal repeat promoter, Rous
Sarcoma Virus, phosphoglycerate kinase or Human
30 Immunodeficiency Virus promoter.

In preferred embodiments, this vector further
includes DNA encoding a mammalian protein under the
transcriptional control of the first promoter.
Preferably, the mammalian protein is ICE, TH, GDNF, or
35 TH. In additional preferred embodiments, this mammalian

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protein is heterologous to the cells that the vector infects.

In various preferred embodiments, the addition of tetracycline either increases (enhances) or inhibits
5 (suppresses) the expression of the mammalian gene.

In preferred embodiments, the second promoter (c) of this vector is selected from the group consisting of SV40 promoter, JC virus promoter, glial fibrillary, nestin, PO, estrogen receptor, and phosphoglycerate
10 kinase. In other preferred embodiments, the third promoter (g) in this vector is CMV promoter or thymidine kinase.

In preferred embodiments, this vector further includes an endonuclease site located in an antisense
15 orientation relative to the DNA encoding the regulator unit. Preferably, this endonuclease site is Xhol, Spel, Sfil, or Bstxl. In preferred embodiments, the selectable marker is puromycin, neomycin, hygromycin or thymidine kinase.
20   In various preferred embodiments, this vector features the 5' retroviral long terminal repeat promoter as the first promoter, the puromycin resistance gene as the selectable marker, the SV40 promoter or JC virus promoter as the second promoter (c), the CMV promoter as
25 the third promoter (g).

In another related aspect, this invention features a method of inducing apoptosis in a cell, including the steps of: (a) providing a tetracycline regulated retroviral vector containing DNA encoding the IL-1 Beta-
30 converting enzyme (ICE), or functional homologues of ICE, under the control of tetracycline; and (b) introducing the vector into the cell. Functional homologues of ICE include, without limitation, FAS, RIP, CPP32, ICE-II, ICE-III, and NEDD-2. In preferred embodiments, the
35 presence of tetracycline either inhibits (suppresses) or

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enhances (increases) the expression of the ICE encoding
DNA.

In yet another related aspect, this invention
features a method of delivering a mammalian regulatable

5 or inducible gene to a human including the steps of: (a)
obtaining cells for implantation; (b) introducing into
the cells of (a) a tetracycline regulated retroviral
vector containing a mammalian gene under the control of
tetracycline; and (c) implanting the cells of (b) into

10 the patient.

In preferred embodiments, the cells in the above
method are obtained from the patient and the presence of
tetracycline either inhibits or enhances (increases) the
expression of the gene. In various preferred

15 embodiments, the gene is ICE, tyrosine hydroxylase, or
glial derived neurotropic factor.

In yet another related aspect, this invention
features a method of delivering a regulatable or
inducible mammalian gene to a mammal including the steps

20 of: (a) obtaining viral particles containing tetracycline
regulated retroviral vectors containing the mammalian
gene; and   (b) infecting cells by administering the
viral particles to an affected organ of the mammal. In
preferred embodiments, the mammalian gene of this method

25 is ICE, tyrosine hydroxylase, or glial-derived
neurotropic factor.

By "operably linked" is meant that a gene and a
regulatory sequence(s) are connected in such a way as to
permit gene expression when the appropriate molecules

30 (e.g., transcriptional activator proteins or proteins
which include transcriptional activation domains) are
bound to the regulatory sequence(s).

By "reporter gene" is meant a gene whose
expression may be assayed; such genes include, without

35 limitation, *lacZ*, amino biosynthetic genes, e.g., the

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yeast *LEU2*, *HIS3*, *LYS2*, *TRP1*, or *URA3* genes, nucleic acid
biosynthetic genes, the mammalian chloramphenicol
transacetylase (CAT) gene, or any surface antigen gene
for which specific antibodies are available. Reporter
5 genes may encode any protein that provides a phenotypic
marker, for example, a protein that is necessary for cell
growth or a toxic protein leading to cell death, or a
protein detectable by a color assay leading to the
presence or absence of color. Alternatively, a reporter
10 gene may encode a suppressor tRNA, the expression of
which produces a phenotype that can be assayed. A
reporter gene according to the invention includes
elements (e.g., all promoter elements) necessary for
reporter gene function.

15   By "promoter" is meant a minimal sequence
sufficient to direct transcription. Also included in the
invention are those promoter elements which are
sufficient to render promoter-dependent gene expression
inducible by the absence or presence of tetracycline *in*
20 *vitro* or *in vivo*; such elements may be located in the 5'
or 3' regions of the gene whose expression is sought to
be controlled.

         By "apoptosis" or "programmed cell death" is meant
a form of cell death in which the cell activates an
25 internal death program. It is generally characterized by
nuclear DNA degradation, nuclear degeneration and
condensation, and the phagocytosis of cell residua.
Apoptosis of tumor cells *in vivo* is evidenced by tumor
cell cytoplasmic reduction, detachment of tumor cells
30 from the tumor architecture, nuclear condensation, and
internucleosomal DNA fragmentation. Apoptosis *in vitro*
is evidenced by cell shrinkage, membrane blebbing, the
release of apoptotic bodies, chromosome condensation and
nuclear blebbing. Apoptosis is to be contrasted with

necrosis which is a form of cell death associated with
external insult to the cell (e.g., poisoning or anoxia).

By "inducible" is meant a DNA sequence whose
expression is mediated by the relative presence or
5 absence of a molecule or compound. The molecule or
compound may interact directly or indirectly with the DNA
sequence expressed, or with another DNA sequence capable
of mediating the expression of the DNA sequence. For
example, in the retroviral Tet system, transactivator
10 hybrid protein (tTA) may induce the expression of a gene
inserted downstream of the tetO-minimal CMV promoter. In
addition, depending upon the arrangement of DNA sequences
within the retroviral TET system, the relative presence
or absence of tetracycline may mediate induction of the
15 tetO-minimal CMN promoter by competitively binding tTA.
For this invention, the terms "drug regulated",
"tetracycline regulated", "drug regulatable",
"tetracycline regulatable", "drug inducible" and
"tetracycline inducible" are used interchangeably.

20   By "tetracycline" is meant tetracycline
$(C_{22}H_{24}N_2O_8)$, a tetracycline derivative or a tetracycline
analogue. Examples of tetracycline derivatives include,
but are not limited to, tetracycline-HCl $(C_{22}H_{25}ClN_2O_8)$,
minocycline, doxycycline, oxytetracycline,
25 chlortetracycline, tetracycline phosphate complex,
tetracycline lauryl sulfate (Laruacycline) and
anhydrotetracycline. For this invention, "tetracycline"
and "tetracycline derivative", or "tetracycline
analogues" are used interchangeably.

30   By "selectable marker" is meant a phenotypic
characteristic by which cells having a particular
genotype may be selected. For example, the presence of a
gene whose expression provides resistance to a particular
antibiotic allows for the selection of cells containing
35 the gene by treating cells with the antibiotic such that

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non-resistant cells are killed. Exampl s of antibiotic
resistance genes include those that conf r resistance to,
without limitation, ampicillin, tetracycline,
chloramphenicol, puromycin, hygromycin, thymidine kinase,
5 and neomycin. In addition to antibiotic resistance
genes, selectable markers may include, without
limitation, suppressor tRNA genes, amino acid
biosynthetic genes, nucleic acid biosynthetic genes, and
genes encoding proteins necessary for cell growth or
10 preventing cell death.

By "ON state" and "OFF state" is meant cellular
conditions under which the expression of a gene is highly
induced and substantially silenced, respectively.
Depending upon the organization and interactions of the
15 DNA sequences which regulate or encode the expression of
the gene product to be delivered, the ON state may be
associated with either the relative presence or absence
of an inducer compound. Similarly, the OFF state may be
associated with the relative presence or absence of an
20 inducer compound. Thus, a continuum of gene expression
exists between the ON state and the OFF state depending
upon the relative amount of inducer compound present.

By "transactivator" is meant an expression product
(i.e., RNA or protein) of a DNA sequence which mediates
25 the expression of another DNA sequence. For example, in
the retroviral Tet system, expression of the DNA sequence
encoding the tetracycline repressor (tetR) fused to the
herpes simplex virus (HSV) transactivator protein, VP16,
produces a transactivator hybrid protein (tTA) which
30 mediates the expression of the tetO-minimal CMV promoter-
gene of interest construct in the response unit. In
addition, RNA transcripts from the DNA sequence encoding
tTA may form duplexes with RNA transcripts from the DNA
sequence encoding the response unit and thereby reduce
35 translation of this transcript.

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Therapeutic Advantages

        As described herein, the invention provides a
number f therapeutic advantag s. As compared to
traditional the dual plasmid Tet systems, a retroviral
5   Tet system vector provides enhanced efficiency of gene
delivery. The retroviral Tet vector also has reduced
constitutive gene expression as compared to the dual
plasmid Tet system. In addition, the precise induction
of gene expression provided by the retroviral Tet system
10 enhances the temporal and quantitative control of gene
product delivery (i.e., the specification of dosage,
frequency, and duration of treatment) and thus, the
vector may be useful in a wide variety of gene therapy
paradigms. Moreover, when directed toward gene therapy
15 for tumors, the retroviral Tet system avoids the
cytotoxicity and lack of specificity characteristic of
other tumoricidal therapies (e.g., ionizing radiation and
chemotherapy).

        The tightly regulated expression afforded by the
20 retroviral Tet system *in vivo* allows time for gene
delivery (e.g., the grafting of packaging cells into the
target and infection of proliferating cells) and
subsequently for the precise induction of death of both
target and packaging cells. Gene transduction efficiency
25 is increased by allowing the packaging cells time to
migrate and infect the target cells, while therapeutic
safety is increased by the concomitant death of target
and packaging cells. For example, when the retroviral
Tet system is used to control the delivery of a late
30 stage apoptosis protein (e.g., ICE) certain mechanisms of
tumor cell resistance may be by-passed (e.g., p53
mutants). Genes known to regulate cell death or cell
cycling may be delivered as a therapeutic agent into
tumor cells by the retroviral Tet system vector.

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Diagnostic Advantages

Similar to its th rapeutic advantage, a retroviral Tet system provides for high efficiency gene delivery of target cells *in vitro* as compared to a traditional dual
5   plasmid Tet system. Likewise, the retroviral Tet system is characterized by reduced constitutive gene expression (i.e., of the response unit) as compared to a dual plasmid Tet system. This feature is particularly useful for the *in vitro* investigation of negative growth
10 influence genes (i.e., cell cycle inhibitors or dominant negative mutants). Investigation of the dose responses of cells to these and other translation products is made possible by the precise regulation of expression of the gene of interest achievable with this system. More
15 specifically, a retroviral Tet system encoding the ICE-*lacZ* fusion protein may provide an *in vitro* model exhibiting synchronized progression toward cell death and thereby enhance the study of the biochemical events associated with apoptosis. In addition, as new cell
20 death and growth suppressive genes and their mechanisms of action are elucidated, these genes may be used in the retroviral Tet system to study the homeostasis of cell proliferation and death.

Coupling inducible retroviral Tet system vectors
25 with the high efficiency ecotropic BOSC23 packaging cells allows for the production of virus after a two day transient transfection and packaging reaction. In addition, the inclusion of the puromycin gene in the retroviral Tet system vectors provides for selection of
30 puromycin resistant producer pools of BOSC23 cells from which increased viral titers may be obtained. Thus, target cells may be infected and the effect of the product of a gene of interest measured in a relatively short period of time. Moreover, because the high viral
35 titer allows for infection of the vast majority of target

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cells, th n d to generate stable cell lines is obviat d
and the potential negative selection f target cells with
low tolerance for the tTA protein is diminished.
Furthermore, retrovirus capable of infecting non-rodent
5 species may be generated by the efficient "ping-pong"
between ecotropic and amphotropic packaging cell lines.

Other features and advantages of the invention
will be apparent from the following description of the
preferred embodiments thereof, and from the claims.

10   Brief Description of the Drawings
        Fig. 1 diagrams the inducible and cell-specific
retroviral cloning vectors used in Examples 1 and 2. All
retroviral vectors contain both components of the Tet
system including the regulator unit [containing the tTA
15 (tetracycline-responsive tansactivator) gene] which is
under the tanscriptional control of internal SV40 or JC
virus promoters, and the response unit which is under the
transcriptional control of a minimal CMV promoter
(lacking enhancers) containing seven tet operators (tetO)
20 Note that the response unit is inserted into the
retroviral vectors in an antisense orientation relative
to the regulator unit.
        A multiple cloning site containing four unique
restriction sites (NotI, PacI, PmeI, BamHI) has been
25 inserted downstream of the response unit to facilitate
the insertion of heterologous genes into the vectors.
The puromycin resistance gene (Puro) is present in all
constructs and is under the transcriptional control of
the 5' retroviral LTR. The retroviral vectors are
30 modular in that both the SV40 promoter and Puro gene can
be removed and replaced by different promoters or
selectable maker genes. Transcription units and
direction of transcription are indicated by arrows. The

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vectors carrying the response gene within the 3' LTR
(c,d; pBPSTR2 and pBPJTR2) are considered double copy
vectors (Hantzopoulos, P A., Sullenger, B. A., G., U. &
Gilboz, E. (1989) *Proc. Natl. Acad. Sci. USA*. 86,
5   3519-3523). The vectors carrying the response unit
between 5' and 3' LTRs are single copy vectors (Panel a),
Panel b): pBPSTR1, pBPJTR1). Abbreviations used: B: Babe
family-based retroviral vector; P: puromycin gene; S:
SV40 early promoter; J: JC virus early promoter; T: tTa;
10   R: response unit. The right side of the figure is the
predicted structure of the retrovirus after integration
into host cell DNA (provirus).

    Fig. 2 is a bar graph showing the effects of the
different internal promoters on gene expression and
15   regulation. NIH 3T3, C6, H56 and Pam212 cells were
infected with either pBPSTlucl (SV40 promoter) or
pBPJTlucl (JC promoter) retrovirus. Ratios of luciferase
activity in the absence (-) and presence (+) of
tetracycline during and after infection reflect inducible
20   regulation (shown above boxes). Values were obtained
from Table 2 and are presented in a graphic manner.

    Fig. 3 (A) is a diagram of the ICE-*lacZ*-containing
tetracycline regulated retroviral vector used in the
experiments. The puromycin resistance gene (Puro) is
25   under the control of the promoter within the 5'-LTR. The
murine ICE-*lacZ* fusion gene is cloned into the response
unit and is under the transcriptional control of *E.
coli*-derived tetracycline resistance operon regulatory
elements (*tetO*) embedded within a minimal CMV promoter
30   (minCMV). The regulator unit encodes a transactivator
hybrid protein (tTA) composed of the tetracycline
repressor (tetR) fused to the herpes simplex virus
transactivator protein, VP16, and is under the

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transcriptional control of the constitutively active SV40
promoter (SV). In the absence of tetracycline, tTA
protein binds *tetO* sequences within the response unit via
its *tetR* domain and activates transcription of the

5 ICE-*lacZ* gene. In the presence of tetracycline, which
binds tTA, tTA dissociates from the response unit and
transcription of the ICE-*lacZ* gene is inhibited. Arrows
indicate the direction of transcription.

   Fig. 3 (B) is a bar graph showing beta -
10 galactosidase enzyme assay. Values were 0.711 ± 0.035 (0
time point; i.e. tetracycline not removed), 4.965 ± 0.094
(12 hours after removal of tetracycline) and 27.046 ± 0.626
(24 hours after removal of tetracycline) milliunits
B-gal/mg protein (means ± sem). Fold induction is shown
15 above each bar.

   Fig. 4 are photographs and a bar graph showing
the induction of apoptosis in 9L-ICE-*lacZ* cells. Panels
A to C: After 24 hours in the presence or absence of
tetracycline, expression of ICE-*lacZ* in 9L-ICE-*lacZ* cells
20 or *lacZ* in 9L-*lacZ* cells, was determined by staining with
X-gal. Panel A: 9L-ICE-*lacZ* cells with tetracycline.
Panel B: 9L-ICE-*lacZ* cells without tetracycline. Panel
C: 9L-*lacZ* cells without tetracycline. Panels D to F:
Viability assay using calcein-AM and propidium iodide.
25 Panel D: 9L-ICE-*lacZ* cells with tetracycline showing
green fluorescence from the cytoplasm of viable cells.
Panel E: 9L-ICE-*lacZ* cells in the absence of tetracycline
for 24 hours, showing intense red fluorescence resulting
from propidium iodide uptake into nuclei of dead cells.
30 Panel F: Magnified view of 9L-ICE-*lacZ* cells without
tetracycline demonstrating rhodamine fluorescence and
showing highly condensed chromatin blebbing outward
through the vesiculated nuclear membrane. Panels G and

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H: Hoescht stain of nuclei. Panel G: 9L-ICE-lacZ cells
with tetracyclin revealing normal nuclei. Panel H:
9L-ICE-lacZ cells in the absence of tetracycline for 24
hours revealing condensed chromatin. Panel I: 9L-
5 ICE-lacZ cells were incubated with and without
tetracycline for 24 hours and cell death was determined
as described above. Percents of dead red cells relative
to all cells counted are noted. Mean percents cell death
were 96.6% ± 0.302 without tetracycline and 1.30 % ±
10 0.446 with tetracycline (means ± sem). Magnifications,
A-C, x100, D-E, x50, F-H, x100.

     Fig. 5 are photographs and a graph showing the
induction of apoptosis in 9L gliosarcoma tumors
containing the ICE-lacZ gene. Panel A to G: 9L-ICE-lacZ
15 cells were stereotactically implanted in the right
striatum in Fisher rats on tetracycline. After
administration of oral tetracycline for 7 days,
tetracycline was removed for the noted time periods, and
the animals were sacrificed thereafter. X-gal
20 histochemistry was performed on coronal brain sections
and counterstained with neutral red. Panel A: Two days
after removal of tetracycline. Edge of 9L-ICE-lacZ tumor
bordering normal brain parenchyma showing individual lacZ
positive cells with rounded apoptotic phenotype. Panel
25 B: Edge of 9L-ICE-lacZ tumor in comparable time point
animal maintained on tetracycline showing absence of lacZ
positive cells. Panel C: Four days after removal of
tetracycline. Whole coronal brain section of animal
showing lacZ positive cells in all regions of the tumor.
30 Note also the areas of cell death, particularly within
the central part of the tumor, but also radiating out
toward peripheral areas. Panel D: Whole coronal brain
section of comparable time point animal maintained on
tetracycline. Note the lack of lacZ positive cells and

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abs nce of areas of cell death. _Panel E:_ Four days after
removal of tetracyclin . Essentially all cells within the
tumor are *lacZ* positive. Also note the nuclear
heterogeneity, typical of apoptotic cells. Panel F: Four
5   days after removal of tetracycline. Apoptotic *lacZ*
positive cells in central area of the tumor and area of
intense cell death. Panel G: Nine days after removal of
tetracycline. *In situ* analysis of DNA fragmentation in
individual cells in area of cell death detected by
10 horseradish peroxidase-digoxigenin nucleotide labeling of
3'-OH DNA ends. Panel H: Subcutaneous growth of
9L-ICE-*lacZ* tumor on and off tetracycline. The group
maintained on tetracycline is shown by black squares and
the group taken off tetracycline is shown by blue
15 circles. There was a significant difference in
subcutaneous tumor growth in animals that were taken off
tetracycline compared to those left on the drug for the
last two measured time points ($P < 0.05$, one-tailed
t-test). Magnifications A-B, x100, E-G, x100.

20   Fig. 6 A is a diagram of the original tetracycline
regulatable retroviral vector, pBPSTR-1, as described in
Example 1. Using this vector the expression of a gene
inserted into the multiple cloning site of the response
unit is dependent on tTA protein binding to tetO
25 sequences in the response unit.

    Fig. 6B is a diagram of the "reverse" tetracycline
regulatable retroviral vector, pBRSrTR-1. Using this
vector, expression of a gene inserted into the multiple
cloning site of the response unit is dependent on
30 "reverse" tTA (rtTA) protein binding to tetO sequences in
the response unit. The reverse tTA (rtTA) protein is
different than the tTA protein present in the original
retroviral vector (pBPSTR-1) because it needs to bind

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tetracycline for it to also bind tetO sequences and
activate expression of a gene in the response unit.

Fig. 7 are diagrams of the GDNF and TH containing
tetracycline regulated retroviral vectors.

5   Fig. 8 is a photograph of the expression of GDNF
and TH, in the presence or absence of tetracycline as
shown by a western blot. Infected and
puromycin-resistant Rat1 fibroblasts clones were examined
for tetracycline dependent regulated expression of the
10 GDNF (Figure 8A, clones GD2, GD3, GD4, GD5, GD6) and TH
genes (see Figure 8B, clones TH1, TH3 and TH4). Shown in
Figures 8A and 8B are western blots of protein from
puromycin resistant clones that had been selected from
pools of Rat1 cells that have been infected with the
15 tetracycline regulatable retrovirus. Clones GD2, GD3,
GD4, GD5, GD6, TH1, TH3, TH4 are different Rat1 clones
selected with puromycin as described in the above
Examples. To assay tetracycline regulation of TH and GDNF
genes in the clones, cells were grown in the presence
20 ("+") or absence ("-") of tetracycline for two days.
Protein lysates were then prepared and equal amounts of
total protein were examined in western blotting analysis
using antibodies against either GDNF (A) or TH (B). "C"
is the non-infected Rat1 control cells.
25

Detailed Description

An inducible or regulatable retroviral vector may
be used to regulate the expression of a gene of interest
which is operably linked to regulatory sequences within
30 the retroviral vector. The vector may be used *in vitro*
or *in vivo* to infect cells in which the controlled
delivery of a gene product is desired. The retroviral
vector includes the Tet system operably linked to a gene

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of interest such that the transcription and translati n
of the gene may be regulated by altering the
concentrati n of tetracycline in cells infected with the
retroviral vector. Using this system, the amount of gene
5 product is proportional to the amount of tetracycline
administered to the cell. Thus, precise control of the
delivery of gene products from a wide variety of genes
may be accomplished.

       In a preferred embodiment, the retroviral vector
10 includes the Tet response unit containing the *teto*
sequence operably linked to a promoter capable of
controlling the transcription of a gene of interest. The
response unit may also include a polylinker, containing
restriction sites unique to the retroviral construct, to
15 facilitate the insertion of genes of interest into the
retroviral vector. In addition, an SV40 polyadenylation
signal may be operatively linked to the gene of interest
to facilitate transcription thereof. Other suitable
polyadenylation signals include, without limitation,
20 AUUAAA, AAUUAA, AAUACA, AAUAAC, and CAUAAA (Wickens, et
al (1984) *Science*, 226:1045-1050). In a preferred
embodiment, the CMV promoter is the cytomegalovirus
promoter (CMV), however, other suitable promoters
include, without limitation, the thymidine kinase
25 promoter. The CMV promoter preferably controls the
expression of a DNA sequence encoding the ICE-*lacZ* fusion
protein, but may also be operably linked to one of many
different other genes of interest, including, without
limitation, ICE, TH, GDNF, and TH.
30   The retroviral vector also includes the Tet
regulator unit which contains the tetR coding sequence
operably linked to the HSV transactivator protein, VP16,
collectively under the control of an SV40 promoter.
Alternatively, the *tetR*-HSV transactivator hybrid protein
35 (tTA) may include transactivator proteins such as *myc*,

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Rb, p53. Moreover, the regulator unit may be
alternatively under the transcriptional control of the
glial fibrillary acidic protein, nestin, P0, estrogen
receptor, phosphoglycerate kinase promoters. In a
5 preferred embodiment, the regulator unit and the response
unit are oriented anti-sense to one another, however,
they may, alternatively, be in a sense orientation. In
addition, the retroviral vector includes the 5' and 3'
LTRs of the Moloney Murine Leukemia virus. Other sources
10 of 5' and 3' LTRs include, without limitation, Rous
Sarcoma Virus (RSV) and Human Immunodeficiency Virus
(HIV). The retroviral vector also includes a mutated
form of the gag gene region to allow for packaging of the
retroviral vector with a packaging cell line such as
15 BOC23 cells. The retroviral vector may also include a
selectable marker such as the puromycin gene.
Alternative selectable markers include, without
limitation, hygromycin, neomycin, thymidine kinase.
Moreover, the retroviral vector may include a second
20 response unit positioned within or outside the 5' or 3'
LTR.

The organization and orientation of the
constituent elements of the retroviral vector may be
varied depending upon the type of control desired. For
25 example, positive control of gene expression (i.e., where
gene product concentration is directly proportional to
inducer concentration) may be accomplished by replacing
the tTA gene in the same transcriptional orientation with
a functionally reverse acting tTA gene. Retroviral
30 vectors organized in this manner are particularly useful
for the intermittent delivery of positive growth factors
(e.g., epidermal growth factor, platelet-derived growth
factor, insulin, growth hormone) because administration
of the inducer compound is only required when gene
35 product delivery is desired. Thus, retroviral vector

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constructs can be designed such that the presence of an
inducer, e.g., t tracyline, enhances transcription
thereby increasing expression of the desired protein
("reverse tTA" or "rtTA"). The "reverse" tetracycline
5 regulated retroviral vector of this invention can be
designed to produce certain desired growth factors,
hormones, growth suppressive factors,
immunoproliferative, immunosuppressive and
immunosupportive factors, and neurotrophic factors that
10 can aid in the maintenance, survival and differentiation
of neuronal and non-neuronal cell types.

Alternatively, negative control of gene product
delivery (i.e., where the gene product concentration is
inversely proportional to inducer concentration) is
15 particularly useful for the delivery of negative growth
factors (e.g., cell death or growth suppressive genes)
because the expression of such genes may be suppressed or
silenced by administration of the inducer compound until
cell death is desired. Genes particularly suitable for
20 use in a negatively controlled retroviral system include,
without limitation, tumor or growth suppressive genes:
p53, Rb, p107, p21, p16, p27 and genes functionally
related to ICE; examples being FAS, RIP, CPP32, ICE-II,
ICE-III, NEDD-2. Such genes may have widespread
25 diagnostic and therapeutic uses.

In general, the drug regulated retroviral vector
of this invention is used to control the expression of
gene transcription. For example, tetracycline can
regulate transcription and therefore the expression of a
30 desired mammalian gene. The tetracycline regulated
retroviral vector of this invention is generally designed
to contain the following components, operationally linked
from 5' to 3': (a) DNA comprising a first promoter; (b)
DNA comprising a second promoter different from the first
35 promoter in (a) and different from the third promoter in

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(e); (c) DNA comprising a tetracycline regulator unit
(tTA) under the transcriptional control of the second
promoter; (d) DNA encoding a tetracycline response unit
(tetO) in an antisense orientation relative to the DNA
5   encoding the regulator unit of (c); and (e) DNA
comprising a third promoter different from first promoter
in (a) and different from second promoter in (b) wherein
said DNA encoding the response unit of (d) is under the
transcriptional control of the third promoter.

10   Listed below are some applications for the drug
regulatable retroviral vectors of this invention in
humans. This list is intended to serve as examples of
different applications using the vectors of this
invention and is not intended to limit the invention
15 whatsoever.

        1)   The drug regulated retroviral vectors of this
invention can be used to deliver and express cytotoxic
gene(s) to a tumor or to a diseased tissue for controlled
killing of the tumor or diseased cells. These cytotoxic
20 gene(s) include, but are not limited to, genes that
encode products that are directly toxic to a specific
tumor or to diseased cells, or those genes which encode
products that are toxic to the tumor or to the diseased
cells, in the presence of a prodrug that requires the
25 product of the delivered gene for activation, by
disrupting any aspect of the tumor or diseased cell
machinery that is essential for tumor or diseased cell
survival. These genes include, but are not limited to,
ICE, bacterial toxins, thymidine kinase and cytosine
30 deaminase.

        2)   The drug regulated retroviral vectors of this
invention can be used to deliver and express therapeutic
gene(s) to a tumor or diseased cells for cytostatic
growth control of the tumor or diseased cells. Examples
35 of therapeutic gene(s) are those genes that encode

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products that can arrest the growth or proliferation of
tumor or diseased cells, but not kill the tumor or
diseased cell. These therapeutic genes includ , but are
not limited to, p53, Rb, p21 and p16.

5   3)   The drug regulated retroviral vectors of this
invention can be used to deliver and express   deliver
of therapeutic gene(s) to diseased cells for replacement
of deficient, defective, or lacking gene product.
Therapeutic gene(s) are those genes that encode products
10 that can replace the product of a gene that is defective
or absent in diseased cells. Examples of therapeutic
genes can be those genes that encode growth factors,
growth suppressive factors, immunoproliferative,
immunosuppressive and immunostimulative factors, and
15 neurotrophic factors that can aid in the maintenance,
survival and differentiation of neuronal and non-neuronal
cell types.

        4)   The drug regulated retroviral vectors of this
invention can be used to deliver and express programmed
20 cell death gene(s) (e.g., genes that induce apoptosis) in
tumor or diseased cells for induction of programmed cell
death in tumor or diseased cells.

        5)   The drug regulated retroviral vectors of this
invention can also be used for *ex vivo* or *in situ*
25 infection and delivery of therapeutic gene(s) to
supportive cells that can be reimplanted into individuals
for the eventual controlled release of the therapeutic
drug(s). Examples of therapeutic genes can be those
genes that encode growth factors, growth suppressive
30 factors, immunoproliferative, immunosuppressive and
immunosupportive factors, and neurotrophic factors that
can aid in the maintenance, survival and differentiation
of neuronal and non-neuronal cell types. This list of
examples is not intended to limit the invention.

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6)   The drug regulated retroviral vectors f this
invention can also be used to deliver therapeutic gene(s)
in utero, *in situ*, or *ex vivo* to human embryonic cells
for replacement of deficient, defective, or lacking gene
5 product(s).

Listed below are some applications for the drug
regulatable retroviral vectors of this invention in the
production of pharmaceuticals or drugs that could benefit
humans or livestock. This list is intended to serve as
10 examples of different applications using the vectors of
this invention and is not intended to limit the invention
whatsoever.

1)   The drug regulated retroviral vector of this
invention can be used to generate eukaryotic cells that
15 can be grown in culture, or transgenic animals, that can
produce therapeutic drug(s) in a controlled manner. Any
gene that can produce a product that has adverse effects
on the eukaryotic producer cells grown in culture, or
transgenic animals, would be a candidate for delivery and
20 regulated expression by the drug regulatable retroviral
vector of this invention.

2)   The drug regulated retroviral vector of this
invention can be used to deliver genes that produce a
product the can increase or decrease the body weight or
25 body fluids of animals used in agribusiness or
agriculture.

3)   The drug regulated retroviral vector can be
used to screen for and isolate novel genes that normally
when expressed in eukaryotic cells are cytostatic or
30 cytoxic (e.g. tumor or growth suppressive genes and
programmed cell death genes). The ability to inhibit the
expression of a cytostatic or cytoxic gene(s) during
screening and isolation will facilitate the isolation of
these types of genes.

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The following examples are presented to
illustrate, without limitation, the invention.

EXAMPLE 1
Self-Contained, Tetracycline Regulated
5   Retroviral Vector System for Gene Delivery
to Mammalian Cells

Tetracycline regulated retroviral vectors were
designed and constructed to contain the tetracycline-
inducible Tet system. The two components of the Tet
10 system were organized within the vectors in a manner that
stringently maintains tetracycline-dependent regulation.

I.   Methods Used in the Following Experiments
        A.   Construction of Retroviral Plasmids
        The retroviral constructs described here are
15 derived from a modified version of pBabePuro which is a
Moloney murine leukemia virus-based vector containing a
puromycin resistance gene under the control of an
internal SV40 promoter (Morgenstern, J. P. & Land, H.
(1990) *Nucl. Acid Res.* 18, 3585-3596; Morgenstern, J. P.
20 & Land, H. (1990) *Nucl. Acid Res.* 18. 1068). The
puromycin resistance gene in pBabePuro was excised with
HindIII and *ClaI* and replaced by the tTA gene. To do
this the entire 1017-bp coding sequence of tTA was
generated by PCR amplification using the pUHD15-1 plasmid
25 (Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci*
USA, 89:5547-5551) as template and primers corresponding
to bp 774-790 (including a *HindIII* restriction site and
the optimal Kozak sequence CCACCATG) and bp 1790-1774
(including a *ClaI* site). This pBabe-based plasmid coding
30 for tTA under the control of the internal SV40 promoter
was designated pBST. To obtain the pBJT plasmid, where
the tTA gene is under the control of the JC promoter, the
SV40 promoter in pBST was removed by excision with *NotI*
and *HindIII*, and replaced by a 299-bp *HindIII/NotI*

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fragment from pCR1000-Mad-1 corr sponding to the JC virus
Mad-1 strain early promoter (Henson, J., Saff r, J. &
Furneaux, H. (1992) *Ann. Neurol.* 32:72-77). The
puromycin resistance gene, isolated as a 660-bp
5   *HindIII/ClaI* fragment from pBabePuro, was then
re-inserted into the *BamHI/NotI* polylinker site of pBST
and pBJT (located 5' to the SV40 and JC promoters) by
blunt-end ligation after filling-in with Klenow, thereby
destroying the *NotI* and *BamHI* sites. The resulting
10 plasmids were designated pBPST and pBPJT, respectively.
        The response plasmid pUHD 10-3 contains the
heptamerized *tetO* sequences, a minimal CMV promoter, a
cloning site for insertion of the gene of interest and an
SV40 polyadenylation signal (Gossen, M. & Bujard, H.
15 (1992) *Proc. Natl. Acad. Sci USA*, 89:5547-5551). To
facilitate cloning of genes into the response cassette
within the retroviral plasmids, the cloning site in pUHD
10-3 was replaced with a polylinker containing
restriction sites unique to the retroviral constructs; to
20 this end, a *SacII-NotI-PacI-PmeI-BamHI* adaptor (5'-
GGCCGCTTAATTAAGTTTAAACG-3' and 5'-
GATCCGTTTAAACTTAATTAAGCGGCCG-3') was ligated into the
SacII/BamHI cloning site of pUHD 10-3. The 857-bp *HinPI*
fragment of this modified response cassette (including
25 *tetO* sequences, minimal CMV promoter, polylinker and SV40
polyadenylation signal) was then inserted into pBPST or
pBPJT in an anti-sense orientation (relative to the tTA
transcription unit) into either the *ClaI* site (located
immediately downstream of the tTA gene; - I series of
30 plasmids) or, using Klenow fill-in and blunt-end
ligation, into the BglII site (located within the U3
region of the 3'-LTR, 36 bp downstream from the 5' end of
the LTR; -2 series of plasmids). The SV40 polyadenylation
signal, derived from the pUHD10-3 plasmid and included in
35 the *HinPI* fragment that had been inserted into the

retroviral plasmids, was removed by excision of a 135-bp
fragment containing the relevant polyadenylation
sequences with *BamHI* and *HpaI,* and replaced by a
BamHI/SmaI adaptor (5'-GATCCCCCGGG-3' and 5'-CCCGGGG-3').
5 Depending on the promoter responsible for transcription
of the tTA gene (SV40 or JC) and the location of the
response unit, the resulting retroviral cloning vectors
were designated pBPSTR1, pBPJTRI, pBPSTR2 and pBPJTR2.
        To construct the retroviral reporter plasmids
10 pBPSTIucI, pBPJTlucl, pBPSTluc2 and pBPJTluc2, the
1700-bp gene for firefly luciferase was excised from
pGEM-luc (Promega, Madison, Wl) with *NotI* and *StuI* and
inserted into the *Notl/PmeI* polylinker site of the four
retroviral cloning vectors.

15   B.   Cell Culture
        The following cell lines were used: rat C6 glioma
cells (American Type Culture Collection (ATCC),
Rockville, MD), mouse NIH 3T3 fibroblasts (ATCC), rat H56
hepatoma cells (Bonifacia, M. J., Ezzeddine, D., Sakaki,
20 Y., Breakefield, X. O. & Saraiva, M. J. (1993) *Neuromusc.*
Disord. 3:275-282), mouse Pam212 squamous carcinoma cells
(Yuspa, J., Saffer, J. & Furneaux, H. (1992) *Ann. Neurol.*
32:72-77), and the ecotropic packaging cell line BOSC23-
(11). C6 glioma cells were grown in Ham's F10 medium
25 (Mediatech, Washington, DC) containing 15% horse serum
(Sigma, St. Louis, MO), 2.5% fetal calf serum (Sigma),
and penicillin G/streptomycin sulfate (100 units/ml and
100 μ/ml, respectively) (Sigma). The other cell lines
were maintained in Dulbecco's modified Eagle's minimal
30 essential medium (Mediatech) supplemented with 10% fetal
calf serum and penicillin G/streptomycin sulfate.

        C.   Transfections, Infections and Determination
              of Viral Titer

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Transfections of BOSC23 cells were performed
essentially as recommended (Pear, W. S., Nolan, G. P.,
Scott, M. L. & Baltimore, D. (1993) *Proc. Natl. Acad.
Sci. USA.* 90:8392-8396). Briefly, 5×10^6 BOSC23 cells
5   were plated in 10 ml medium per 100-mm dish. After 24
hours, the medium was replaced with 5 ml fresh medium.
After an additional 3 hours, cells were transfected with
15 μg retroviral plasmid DNA using the calcium phosphate
precipitation technique. Just prior to transfection,
10 chloroquine was added to a final concentration of 25 μM.
Six hours post-transfection, the cells were washed twice
and 10 ml medium was added. Twenty-four hours
post-transfection, the medium was removed and 5 ml of
fresh medium was added. The medium containing the virus
15 was removed 48 hours post-transfection, filtered through
a 0.45-μm filter and either used directly or stored
frozen until use. Media from different plates containing
BOSC23 cells transfected with the same retroviral
construct were pooled to provide a common stock of viral
20 titer for each set of experiments. BOSC23 cells were
grown in either the presence (1 μg/ml, Sigma) or absence
of tetracycline during packaging of virus.

Cells which had been plated at a density of 5×10^5
per 100-mm dish the day before infection, were infected
25 in the presence of polybrene (4 μg/ml, Sigma) with 5 ml
of filtered medium containing virus. After 4 hours, the
medium was aspirated and fresh medium was added. Cells
were washed twice 20 hours post-infection and luciferase
assays were performed 48 hours post-infection. To study
30 tetracycline-dependent regulation of gene expression,
cells were grown in the presence (1 μg/ml) or absence of
tetracycline from 3 hours before infection until
harvesting. All experiments were performed at least
twice in duplicate.

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For titering, NIH 3T3 cells were infected with
serial dilutions f filtered BOSC23 supernatant. Cells
were split 1:10 into selection medium containing
puromycin (1.5 µg/ml, Sigma) 48 hours post-infection.
5 Fresh media was added every 3 days, and colonies were
counted 12 days later after fixation and Giemsa staining.

D.   Luciferase Assay
Cells were washed three times in phosphate
buffered saline and then lysed in 1 mM dithiothreitol/1%
10 Triton X-100/luciferase buffer (25 mM glycylglycine, 15
mM $MgSO_4$, 4 mM EGTA, pH 7.8) at 4°C. Cell lysates were
collected from plates with a cell scraper and centrifuged
for 4 min at 7,800 x g. Aliquots (100 µl) of the
supernatant were mixed with 370 µl luciferase buffer
15 containing 2 mM ATP, I mM dithiothreitol and 15 mM
potassium phosphate, pH 8.3, and assayed for luciferase
activity in a 1251 luminometer (Wallac, Gaithersburg, MD)
using the integral mode. D-Luciferin (Sigma) was used at
a saturating substrate concentration (0.2 mM in
20 luciferase buffer). Luciferase activity was adjusted to
protein content of the lysates, as determined according
to Lowry.

II.   Results
A.   Construction of Retroviral Vectors.
25   To incorporate the tetracycline inducible system
into a retroviral vector, a retroviral vectors containing
the tTA gene under the control of either the broadly
active SV40 promoter or the glial specific JC virus
promoter was first constructed (Feigenbaum, L., Khalili,
30 K., Major, E. & Khoury, G. (1987) *Proc. Natl. Acad. Sci.*
USA. 84 3695-3698;
Tada, H., Lashgari, M., Rappaport, J. & Khalili, K.
(1989) *J. Virol.* 63, 463-466) (regulator unit). An

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important consideration for efficient regulation of a
gene inserted into the response unit is where to insert
this unit relative to the promoters within the regulator
unit and the 5'-LTR of the virus. For the Tet system to
5 function efficiently, the response unit must operate
independently of other promoters within the virus. Two
different cloning strategies were used to determine the
optimal placement of the response unit within the
retroviral vectors. In all of these constructs the
10 luciferase gene was inserted into the response unit to
allow quantitation of tetracycline- dependent regulation.

    In one strategy the response unit was cloned into
a *ClaI* site, located immediately downstream of the 3' end
of the regulator unit, in an opposite orientation to the
15 tTA -containing regulator unit (Fig. 1a,b; pBPSTR1 and
pBPJTR1). In this orientation, expression of the coding
strand of a gene within the response unit can only be
directed from the response unit promoter (tetO/minimal
CMV) and not from the promoters in the regulator unit or
20 viral 5'-LTR. Another advantage of inserting the
response unit in this antisense orientation is that, in
principal, a small amount of transcript produced due to
potential low level leakiness of the response unit
promoter should form RNA duplexes with the vast excess of
25 transcripts originating from the constitutively active
promoters within the regulator unit and 5'-LTR.

    A second strategy was to clone the response unit
into a *BgIII* site within the U3 region of the 3'-LTR
(Fig. 1c,d, pBPSTR2 and pBPJTR2), again in the antisense
30 orientation relative to the regulator unit. These
vectors are considered "double copy" vectors
(Hantzopoulos, P A., Sullenger, B. A., G., U. & Gilboz,
E. (1989) *Proc. Natl. Acad. Sci. USA.* 86, 3519-3523),
because during replication and integration of the
35 retrovirus, the viral 3'-LTR is duplicated and replaces

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the viral 5'-LTR that is present in the plasmid form of
the vector.

Thus, after replication and integration, any gen
cloned into the U3 region of the 3'-LTR will ultimately
5 be located 5' to the promoter within the U5 region of the
LTRs. The logic for this cloning strategy is that a gene
regulated by the tetO/minimal CMV promoter unit will not
be transcribed by the promoters within the viral 5'-LTR
or regulator unit (see Fig. 1c, d).

10   B.   Regulation of Luciferase Activity Using
                  Single-Copy Versus Double-Copy Vectors and
                  Different Packaging Conditions

           Using NIH 3T3 cells as target cells, tetracycline-
dependent regulation of luciferase activity was observed
15 for all retroviral constructs. Variability in the
regulation of luciferase activity was observed depending
on (1) whether tetracycline was present during packaging
of the retrovirus in BOSC23 cells and (2) the
organization of the components of the Tet system within
20 the vectors (Table 1). When pBPSTluc1 virus were
packaged in BOSC23 cells either in the presence (1 ug/ml)
or absence of tetracycline, and then used to infect NIH
3T3 cells plated either in the presence (1 ug/ml) or
absence of tetracycline, luciferase activity was 321-fold
25 or 179-fold lower than in the absence of tetracycline.
This vector thus allows efficient transfer of the
components of the Tet system into target cells, including
stringent repression of gene expression by tetracycline
and >100-fold differences between ON and OFF states
30 (Table 1).

           To examine whether the presence of one or two
copies of the response gene within the provirus affects
regulation, luciferase activity was compared in NIH 3T3
cells infected with either the single-copy vector
35 pBPSTluc1 or the double-copy vector pBPSTluc2. When

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tetracycline was present during the packaging of
retroviral constructs in BOSC23 cells, but absent during
infection of NIH 3T3 cells, luciferase activity with the
single-copy vector was about 4-fold higher than that
5 obtained with the double-copy vector (Table 1). When
tetracycline was present during infection of NIH 3T3
cells, repression of luciferase activity appeared similar
for both the single-copy and double-copy vectors;
however, taking into account the induced activity in the
10 double-copy vector is about 4-fold lower than the
single-copy vector and that there is a corresponding
increase in repressed activity as induced activity
increases (for example, compare values for NIH 3T3 cells
infected with pBPSTluc1 in Tables 1 and 2), the true
15 magnitude of repressed luciferase activity in the
double-copy vector should also be correspondingly 4-fold
higher.

Tetracycline-dependent regulation of luciferase
activity after packaging in the presence of tetracycline
20 was 321-fold with the single-copy vector, but only
49-fold with the double-copy vector. When tetracycline
was absent during packaging of retrovirus, regulation
appeared to be lower, but single-copy vectors still
showed better regulation than double-copy vectors
25 (179-fold versus 18-fold, Table 1). Because of the
better regulation of the Tet system observed with virus
that had been packaged in BOSC23 cells in the presence of
tetracycline, in all subsequent experiments we used virus
packaged in this manner.

30   C.   Regulation of Luciferase Activity by
            Different Internal Promoters Controlling tTA
            Expression
        To examine whether cell type-specific expression
could be introduced into the Tet system-containing
35 retroviral vectors, the glial specific JC virus early

promoter was used to control expression of the tTA gene,
in ord r to evaluat whether luciferase activity could be
restricted to cells of glial origin.

      pBPSTluc1 or pBPJTluc1 retrovirus were used to
5 infect cell lines derived from different tissue origins:
NIH 3T3, mouse fibroblasts; C6, rat glioblastoma; H56,
rat hepatoma and Pam212, mouse squamous carcinoma. In
glial derived C6 cells infected with the pBPJTluc1
retrovirus, induced luciferase activity was higher than
10 the activity observed with the pBPSTluc1 retrovirus (Fig.
2, Table 2). In contrast, in all non-glial cell types,
induced luciferase activities were higher when using the
pBPSTluc1 virus than the luciferase activity observed
when using the pBPJTluc1 virus (Fig. 2, Table 2).
15   Regulation and induced luciferase activity were
generally higher in the mouse cell lines (NIH 3T3,
Pam212) than in the rat cell lines (C6, H56), probably
due to higher infectability of the mouse cells with
ecotropic virus (Fig. 2, Table 2). The highest
20 tetracycline-dependent regulation was observed in NIH 3T3
cells with an induction of 336-fold (regulation of the
other cell types varied from 11 to 127-fold).

      D.   Viral Titers
      In transient packaging reactions using the BOSC93
25 cells, the single-copy vector pBPSTluc1 produced about
ten-fold higher viral titers than the double-copy vector
pBPSTluc2 (Table 1). To examine whether transcriptional
interference occurs in the retroviral vectors due to the
placement of transcriptional units in opposing
30 orientations to each other, BOSC23 cells were grown in
the presence or absence of tetracycline during packaging
of pBPSTluc1 and pBPSTluc2 constructs. Because
tetracycline causes tTA to dissociate from tetO sequences
and thereby inhibiting transcription of the response

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unit, the presence of tetracycline may facilitate the
production of genomic transcripts and increase the number
of viral particles. However, the titer did not
significantly increase after packaging in the presence of
5 tetracycline (Table 1). No differences in the viral
titer were observed between the pBPSTluc1 and pBPJTluc1
virus or pBPSTluc2 and pBPJTluc2 (data not shown).

III.   Summary
        A tetracycline regulated retroviral vectors was
10 designed and constructed to contain the tetracycline
inducible Tet system. The two components of the Tet
system were organized within the vectors in a manner that
stringently maintains tetracycline-dependent regulation.
        Regulated expression of an indicator gene inserted
15 into the retroviral vectors was examined in several
different cell types. In infected NIH 3T3 cells, levels
of induction in the absence of tetracycline were observed
to be as high as 336-fold over levels in the presence of
tetracycline, which were extremely low. Tetracycline
20 dependent regulation was observed in all other transduced
cell types and ranged from 24 to 127-fold. The
generation of retroviral vectors containing regulatory
elements that allow for the regulated expression of
heterologous genes and that have the ability to infect
25 virtually all dividing target cells should greatly
facilitate the biochemical and genetic examination of a
broad range of genes.
        One novel feature of the retroviral vectors are
that the Tet system was organized within a retroviral
30 vector so that high levels of constitutively produced tTA
mRNA function not only for production of tTA protein, but
also to decrease basal expression of the response unit by
apparent antisense inhibition. This antisense inhibition
is evident when comparing tetracycline dependent

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regulation of the single and double copy vectors. For
example, in NIH 3T3 cells, a 321-fold induction of
luciferase activity was observed in the single copy
vector pBPSTlucI where as a 49-fold induction was

5   observed in the double copy vector pBPSTluc2. The lower
induction value observed for the double copy vector is
due to higher basal expression in the OFF state (plus
tetracycline) and lower induced expression in the ON
state (minus tetracycline) compared to activities

10 observed for the single copy vector. Although basal
expression of the response unit within the 3'-LTR of the
double copy vector is subject to antisense inhibition,
the response unit within the 5'-LTR is not. Thus, the
higher basal expression observed in the double copy

15 vector is from the response unit within the 5'-LTR. The
true magnitude of the antisense effect in the single copy
vector is probably higher than the data shown in Table 1,
because of the lower titers of the double copy vector.
The basal luciferase activity of the single copy vector

20 is therefore much lower than that expected on the basis
of gene dosage, which is probably the consequence of
antisense inhibition. Interestingly, although it would
be expected that antisense inhibition would also occur
when transcription in the response unit is activated with

25 tTA binding, this apparently is not a problem since
relatively high levels of luciferase activity are
detectable in the ON state.

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Table 1. Regulated expression of luciferase in NIH 3T3 cells using retroviral vectors with self-contained tetracycline-dependent inducible elements

Vector	Tet During Packaging	Tet During Infection	Luciferase Activity	Fold Induction	Titer (CFU/ml)
pBPSTluc1 pBPSTluc1	+ +	- +	91,082 284	321	2.1×10^5
pBPSTluc1 pBPSTluc1	- -	- +	83,671 467	179	1.3×10^5
pBPSTluc2 pBPSTluc2	+ +	- +	21,412 434	49	2.7×10^4
pBPSTluc2 pBPSTluc2	- -	- +	21,530 1,174	18	2.4×10^4

Retroviral vectors, pBPSTluc1 and pBPSTluc2 containing the luciferase gene under the transcriptional control of the tetracycline responsive transactivator (tTA) were transiently transfected into BOSC23 packaging cells (two days). Tetracycline was present (+) or absent (-) during packaging. Packaged retroviral particles were used to infect target NIH 3T3 cells. NIH 3T3 cells were plated in the presence or absence of tetracycline for measurement of inducible luciferase activity. Luciferase activity is expressed as relative light units per mg total protein cell lysate. Fold induction is the ratio -/+ tetracycline. Experiments were performed in duplicate and confirmed in additional experiments performed in an identical manner. Serial dilutions of media from packaging cells were used for titering of virus on NIH 3T3 cells. Infected cells were placed under puromycin selection and resistant colonies were counted.

Table 2. Regulated expression of luciferase in different cell types using retroviral vectors with self-contained tetracycline-dependent inducible elements

Cell Type	Vector	Tet During Infection	Luciferase Activity	Fold Induction
NIH 3T3	pBPSTluc1	−	263,190	
"	pBPSTluc1	+	784	336
"	pBPJTluc1	−	91,714	
"	pBPJTluc1	+	669	137
C6	pBPSTluc1	−	66,347	
"	pBPSTluc1	+	2,787	24
"	pBPJTluc1	−	76,713	
"	pBPJTluc1	+	2,271	34
H56	pBPSTluc1	−	86,970	
"	pBPSTluc1	+	3,014	29
"	pBPJTluc1	−	25,783	
"	pBPJTluc1	+	2,267	11
Pam212	pBPSTluc1	−	512,346	
"	pBPSTluc1	+	4,039	127
"	pBPJTluc1	−	201,830	
"	pBPJTluc1	+	5,490	37

Retroviral vectors containing the luciferase gene under the transcriptional control of the tetracycline-responsive transactivator (tTA) were transiently transfected into BOSC23 cells and packaged in the presence of tetracycline. tTa is under control of the SV40 promoter in pBPSTluc1 or the JC promoter in pBPJTluc1. Packaged retroviral particles were used to infect NIH 3T3 fibroblasts, C6 glioma cells, H56 hepatoma cells and Pam212 squamous carcinoma cells. During and after infection, NIH 3T3 cells were plated in the presence (+) or absence (-) of tetracycline for measurement of regulatable luciferase activity. Luciferase activity is expressed as relative light units per mg protein cell

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lysate. Fold induction is the ratio of luciferase activity -/+ tetracycline. Experiments were performed in duplicate and confirmed in additional experiments performed in an identical manner.

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EXAMPLE 2
Drug Regulated Apoptotic Death in a
Rat Glioma Model Mediated by a
Novel Retroviral Vector

5   This Example demonstrates in vivo efficacy of the
tetracycline regulated retroviral vector. In this
Example, a murine ICE-*lacZ* fusion gene was introduced
into a novel retroviral vector designed to achieve
regulated ectopic expression of a foreign gene in
10 mammalian cells.

I.   Introduction

IL-1B-converting enzyme (ICE) is a member of a
growing family of cysteine proteases shown to be a
crucial component in the activation of a genetic program
15 that leads to autonomous cell death in mammalian cells.
ICE is a cysteine protease that processes pro-IL-1B to an
active form (Cerretti, D.P., *et al*. Molecular cloning of
the interleukin-1 beta converting enzyme. *Science*.
256:97-100 (1992); Thornberry, N.A., *et al*. A novel
20 heterodimeric cysteine protease is required for
interleukin-1 beta processing in monocytes. *Nature*.
356:768-74 (1992). ICE is a mammalian homolog of the *C.
elegans* cell death gene, ced-3 (Yuan, J., Shaham, S.,
Ledoux, S., Ellis, H.M., & Horvitz, H.R. The *C. elegans*
25 cell death gene ced-3 encodes a protein similar to
mammalian interleukin-1 beta-converting enzyme. *Cell*.
75:641-52 (1993)) and its over expression induces
programmed cell death in Rat-1 fibroblasts (Miura, M.,
Zhu, H., Rotello, R., Hartwieg, E.A., & Yuan, J.
30 Induction of apoptosis in fibroblasts by IL-1
beta-converting enzyme, a mammalian homolog of the *C.
elegans* cell death gene ced-3. *Cell*. 75:653-60 (1993).
Therapies that restore the ability to undergo programmed
cell death may be of considerable benefit in some
35 malignancies (Thompson, C.B. Apoptosis in the

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pathogenesis and treatment of disease. *Science*,
267:1456-62 (1995).

Initiation of programmed cell death as a means of
tumoricidal therapy for gliomas was studied by delivering
5 the ICE gene under the control of a drug regulated
promoter in a retroviral vector. This approach initiates
a physiologic cascade of events that leads to apoptosis.
The novel drug regulated retroviral vector, as described
in Example 1, allows precise control of the expression of
10 this cell death gene. By killing glioma cells through a
genetic mechanism, this strategy avoids the cytotoxicity
and lack of specificity of ionizing radiation and
chemotherapeutic agents, both of which have not
significantly altered the natural history of this disease
15 (Loeffler, J.S. Radiotherapy in managing malignant
gliomas: current role and future directions. *Advances
in Oncology*. 8:14-20 (1992); Kyritsis, A.P. & Levin, V.A.
Chemotherapeutic approaches to the treatment of malignant
gliomas. *Advances in Oncology*, 8:9-13 (1992).
20   To achieve tight control of expression of a
programmed cell death gene, an ICE-*lacZ* fusion gene,
which has previously been shown to cause apoptosis of
cells in culture (Miura, M., Zhu, H., Rotello, R.,
Hartwieg, E.A., & Yuan, J. Induction of apoptosis in
25 fibroblasts by IL-1 beta-converting enzyme, a mammalian
homolog of the *C. elegans* cell death gene ced-3. *Cell*.
75:653-60 (1993), was introduced into a novel drug
regulated retroviral vector (Paulus, W., Baur, 1., Boyce,
F.M., Breakefield, X.O., & Reeves, S.A. Self contained,
30 tetracycline regulated retroviral vector system for gene
delivery to mammalian cells. *J Virol*. In press). The
ICE-*lacZ* fusion gene corresponded to the biologically
active form of the murine ICE protein (consisting of the
P20 and P10 subunits) and the *E. coli lacZ* gene (Miura,
35 M., Zhu, H., Rotello, R., Hartwieg, E.A., & Yuan, J.

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Induction of apoptosis in fibroblasts by IL-1
beta-converting enzyme, a mammalian homolog of the *C.*
elegans cell death gene ced-3. *Cell.* 75:653-60 (1993).
The tetracycline-responsive promoter allows for
5 inhibition of transcription of the ICE-*lacZ* fusion gene
in the presence of drug, but allows transcription to
proceed in the absence of the drug (Gossen, M. & Bujard,
H. Tight control of gene expression in mammalian cells by
tetracycline-responsive promoters. *Proc Natl. Acad. Sci.*
10 *U S A.* 89:5547-51 (1992)).

II.   Methods Used
        A.   Construction of Drug Regulated Recombinant
             Retrovirus
        The tetracycline regulated aspect of the
15 retroviral vector is conferred by the response and
regulator units of the previously described tetracycline
inducible promoter system (Gossen, M. & Bujard, H. Tight
control of gene expression in mammalian cells by
tetracycline-responsive promoters. *Proc Natl Acad Sci U*
20 *S A.* **89** 5547-51 (1992)). The retroviral vector backbone
is a modified version of the Moloney murine leukemia
virus-based pBABE vector (Morgenstern, J.P. & Land, H.
Advanced mammalian gene transfer: high titre retroviral
vectors with multiple drug selection markers and a
25 complementary helper-free packaging cell line. *Nucleic*
Acids Res. 18:3587-96 (1990)) and contains the puromycin
resistance gene (Puro) under the control of the promoter
within the 5'-LTR. A full description of the
construction of the tetracycline regulated retroviral
30 vector is described elsewhere (Paulus, W., Baur, 1.,
Boyce, F.M., Breakefield, X.O., & Reeves, S.A. Self -
contained, tetracycline regulated retroviral vector
system for gene delivery to mammalian cells. *J Virol.* in
press). The regulator unit encodes a transactivator
35 hybrid protein (tTA) constitutively driven by the SV40

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promoter. The murine ICE-*lacZ* fusion gene was excised
from BSM10Z4. This plasmid was digested with *XhoI* and
filled-in by T4 DNA polymerase, then digested with *NotI*,
and this fragment was cloned into the *NotI-PmeI* sites in
5   the polylinker of the retroviral vector pBSTR-19.
Inserting the ICE-*lacZ* fusion gene within the response
unit in this manner places the gene under the
transcriptional control of *E. coli*-derived tetracycline
resistance operon regulatory elements *(tetO)* embedded
10 within a minimal CMV promoter (minCMV). To construct the
control *lacZ* retrovirus, a *HindIII/EcoRI* fragment was
excised from pactβgal' and filled-in with T4 DNA
polymerase in the presence of deoxynucleotide
triphosphates (dNTPs) and then cloned into the *PmeI* site
15 of pBSTR-1.

        B.   Cell Culture
        The drug regulated retroviral vector containing
the ICE-*lacZ* gene was transfected into BOSC23 packaging
cells in the presence of tetracycline to produce
20 infectious retrovirus as described (Pear, W.S., Nolan,
G.P., Scott, M.L., & Baltimore, D. Production of high -
titer helper-free retroviruses by transient transfection.
Proc Natl Acad Sci U S A. 90, 8392-6 (1993)). Rat 9L
gliosarcoma cells (ATCC) were then infected with the
25 supernatant containing retrovirus and cloned under
puromycin selection (5μg/ml) in the presence of
tetracycline (1μg/ml) for approximately 10 days. A
puromycin-resistant clone that expressed a high amount of
ICE-*lacZ* in the absence of tetracycline, designated
30 9L-ICE-*lacZ*, was further characterized.

        C.   B-Galactosidase Assay
        9L-ICE-*lacZ* was characterized for the induction of
the ICE-*lacZ* gene using a B-galactosidase assay which

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measures the am unt of th substrat ONPG
(o-nitrophenyl-B-D-galactopyranoside) that is hydrolyzed
to onitroph nol by absorbance at 420 nm (Promega).
9L-ICE-*lacZ* cells were seeded at a density of 2 X

5   105/well in 6-well dishes and grown in media (Dulbecco's
    modified Eagle's medium with 10% fetal calf serum)
    containing tetracycline (1 μg/ml). The next day, cells
    were washed and placed in media with or without
    tetracycline (1 μg/ml). Cells grown in the presence or

10 absence of tetracycline for 12 and 24 hours were
    harvested and assayed for B-galactosidase activity and
    total protein content (Bradford assay). Experiments were
    done in triplicate and all assays were performed in
    duplicate.

15   D.   Histological Analysis of B-galactosidase
                  Expression
                  Cells were seeded at a density of 2 X 10^5/well in
    6-well dishes. After 24 hours in the presence or absence
    of tetracycline, expression of ICE-*lacZ* in 9L-ICE-*lacZ*

20 cells or *lacZ* in 9L-*lacZ* cells, was determined by fixing
    the cells with 0.5% glutaraldehyde for 10 min, rinsing
    three times with phosphate-buffered saline (PBS), and
    staining in X-gal buffer (1 mg/ml 5-bromo-4-chloro-3-
    indoxyl B-galactoside, 5 mM K_3Fe(CN)6, 5 mM

25 K_4Fe(CN)$_6$-3H$_2$0, 2 mM MgCl$_2$ in 0.1 M sodium phosphate
    buffer (pH 7.4) at 37°C for 12 hours (Price, J., Turner,
    D., & Cepko, C. Lineage analysis in the vertebrate
    nervous system by retrovirus-mediated gene transfer.
    Proc. Natl. Acad. Sci. U.S.A. 84:156-160 (1987)).

30   E.   Viability Assay
                  9L-ICE-*lacZ* cells were grown on coverslips and 24
    hours after ICE-*lacZ* induction by removal of
    tetracycline, culture medium was replaced with 1 μM
    calcein-AM and 10 μg/ml propidium iodide in PBS for 20

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min at 37°C. Viable c lls cleave calcein-AM to calcein,
producing green flu rescence, whereas dead cells take up
propidium iodide into the nucleus, resulting in intense
red fluorescence. Green and red fluorescence were
5 simultaneously visualized using a Nikon transmission
microscope (Asai, A., et al. Negative effects of
wild-type p53 and c-Myc on cellular growth and
tumorigenicity of glioma cells. Implication of the tumor
suppressor genes for gene therapy. *J. Neuro-Oncol.* 19,
10 259-268 (1994); Louis, D.N. The p53 gene and protein in
human brain tumors. *J. Neuropath. and Exper. Neurol.* 53,
11-21 (1994)). Three 0.8 mm^2 fields were counted for
each 50 mm well. All wells were counted in triplicate
and over 300 cells were scored per well. Percents of
15 dead red cells relative to all cells

     F.   Hoescht Stain
          9L-ICE-*lacZ* cells grown on cover slips were
prefixed with 4% paraformaldehyde and washed in PBS,
incubated with Hoescht 33258 dye (final concentration, 5
20 μM) for 1 min, washed again in PBS, and examined with a
Zeiss Axiophot fluorescence microscope.

     G.   9L-ICE-lacz Expression in Intracranial Rat
          Glioma Model
          12 Male CD Fisher rats weighing approximately 300
25 g (Charles River) were administered tetracycline (1.0
mg/ml) in their drinking water as described (Fishman,
G.J., Kaplan, M.L., & Buttrick, P.M. Tetracycline
regulated cardiac gene expression *in vivo. J Clin Invest.*
93:1864-8 (1994)). Four days later, animals were
30 anesthetized with ketamine/xylazine (1/1) and 2 X 10^5
9L-ICE-*lacZ* cells were stereotactically implanted in the
right striatum. The implantation procedure was
essentially as described previously (Kobayashk N., AHen,
N., Clendenon, N.Rv & Ko, L.W. An improved rat brain-

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tumor model. *J Neurosurg*. 53:808-15 0980)). Eight
animals wer taken off tetracycline 7 days later and
sacrificed at 2, 4, 6, and 9 days post removal of
tetracycline and perfused with 4% paraformaldehyde,
5   sectioned, and stained for X-gal histochemistry as
described above and counterstained with neutral red.
Comparable time point 9L-ICE-*lacZ* tumor-bearing animals
maintained on tetracycline were sacrificed in parallel
with the animals that had been taken off tetracycline.
10 *In situ* DNA end-labeling was performed on frozen brain
sections of 9L-ICE-*lacZ* tumor-bearing animals on or off
tetracycline for 4 or 9 days. Internucleosomal DNA
fragmentation was detected by horseradish peroxidase-
digoxigenin nucleotide labeling of 3'-OH DNA ends
15 (Apoptag, Oncor). All animal studies were done in
accordance with guidelines for animal care by the
Massachusetts General Hospital Subcommittee on Animal
Care.

        H.   Subcutaneous Growth of 9L-ICE-*lacZ* Tumor on
20   and off Tetracycline
        Eight animals were given tetracycline in their
drinking water for 4 days and then 5 X 10^5 9L-ICE-*lacZ*
cells in a total volume of 0.1 ml were injected
subcutaneously in the right flank of each rat. Animals
25 were maintained on tetracycline for 7 days at which point
half the animals were then taken off the drug. Tumor
volumes were determined at two day intervals with the aid
of calipers.

III.   Results
30   A.   Induction of ICE-lacZ Expression Using a Drug
              Regulated Retroviral Vector
        The drug regulatable aspect of the retroviral
vector was introduced by including both the "response"
and "regulator" units of the previously described

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tetracycline (Tet) inducible promoter system (G ssen, M. & Bujard, H. Tight c ntrol of g n expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A.* 89:5547-51 (1992)) into the
5 backbone of a MoMLV-derived retrovirus vector (Fig. 3A). Fig. 3 (A) is a diagram of the ICE-*lacZ*-containing tetracycline regulated retroviral vector used in the experiments. The puromycin resistance gene (Puro) is under the control of the promoter within the 5'-LTR. The
10 murine ICE-*lacZ* fusion gene is cloned into the response unit and is under the transcriptional control of *E. coli*-derived tetracycline resistance operon regulatory elements (*tetO*) embedded within a minimal CMV promoter (minCMV). The regulator unit encodes a transactivator
15 hybrid protein (tTA) composed of the tetracycline repressor (tetR) fused to the herpes simplex virus transactivator protein, VP16, and is under the transcriptional control of the constitutively active SV40 promoter (SV). In the absence of tetracycline, tTA
20 protein binds *tetO* sequences within the response unit via its *tetR* domain and activates transcription of the ICE-*lacZ* gene. In the presence of tetracycline, which binds tTA, tTA dissociates from the response unit and transcription of the ICE-*lacZ* gene is inhibited. Arrows
25 indicate the direction of transcription.

Extremely low levels of basal expression from the response unit in the presence of tetracycline were accomplished (Paulus, W., Baur, 1., Boyce, F.M., Breakefield, X.O., & Reeves, S.A. Self contained,
30 tetracycline regulated retroviral vector system for gene delivery to mammalian cells. *J Virol.* in press) by placing the response and regulator units of the Tet inducible system in an antisense configuration (Fig. 3A).

Rat 9L gliosarcoma cells were infected with the
35 retroviral vector containing the ICE-*lacZ* gene and cloned

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under puromycin selection in the continued presence f
tetracycline. Multiple 9L puromycin-resistant clones
were isolated and one of the clones, that stain d
intensely with X-gal when tetracycline was removed, was
5 designated 9L-ICE-*lacZ* and further characterized for
tetracycline-dependent expression of the ICE-*lacZ* gene.
Using an B-galactosidase enzyme assay, induction of
ICE-*lacZ* gene expression in this 9L-derived clone was
7.99-fold and 38.1-fold, 12 hours and 24 hours,
10 respectively, after removal of tetracycline (Fig. 3 B).

          B.   Regulated Expression of ICE-*lacZ* Induces
                 Apoptosis in Cultured Glioma Cells

      9L-ICE-*lacZ* cells grown in the presence of
tetracycline did not stain blue with X-gal and exhibited
15 normal morphology (Fig. 4A). In contrast, X-gal staining
of 9L-ICE-*lacZ* cells 24 hours after removal of
tetracycline demonstrated that all cells stained blue,
and thus were expressing the ICE-*lacZ* gene (Fig. 4B). In
addition to blue staining, all cells exhibited typical
20 morphologic features of apoptosis, including cell
shrinkage, membrane blebbing, and apoptotic bodies (Fig.
4B). A clone of 9L cells, 9L-*lacZ*, infected with a
control vector containing the *lacZ* gene under the same
tetracycline-dependent promoter stained blue without any
25 apoptotic features in the absence of tetracycline (Fig.
4C).

      Cell viability was assessed by a calcein-AM/
propidium iodide double-fluorescence assay. Viable
9L-ICE-*lacZ* cells grown in the presence of tetracycline
30 exhibited green cytoplasmic fluorescence (Fig. 4D), while
dead cells taken off tetracycline for 24 hours exhibited
red nuclear fluorescence (Fig. 4E). Propidium iodide
fluorescence revealed a characteristic nuclear blebbing
pattern in all cells when tetracycline had been removed
35 for 24 hours (Fig. 4F). Ultraviolet absorption after

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Hoechst staining of cells on tetracycline exhibited
large, normal nuclei (Fig. 4G), whereas cells off
tetracycline exhibited chromatin condensation and
apoptotic morphology in their nuclei (Fig. 4H).

5   In the absence of tetracycline for 24 hours, 96.6%
of cells had undergone programmed cell death while only
1.3% of cells were dead in a parallel culture maintained
with tetracycline (Fig. 4I).

        C.   Regulated Expression of ICE-lacZ Induces
10   Apoptosis In an Intracranial Glioma Model
        To determine whether control of ICE expression and
induction of apoptosis could be achieved in vivo,
9L-ICE-lacZ cells were implanted in the right striatum of
rats that had been on tetracycline for 4 days. To allow
15 initial growth of the implanted tumor cells, animals were
given tetracycline hydrochloride for 7 days in their
drinking water. The animals were then divided into two
groups; in one group, tetracycline was retained in their
drinking water, while in the other group, tetracycline
20 was removed. Animals from both groups were then
sacrificed at 2, 4, 6, and 9 days after removal of
tetracycline. Animals at 2 days after removal of
tetracycline showed X-gal staining particularly in tumor
cells adjacent to normal brain with rounded blue cells
25 reminiscent of the condensed cytoplasm associated with
apoptosis (Fig. 5A). Animals of a comparable time point
maintained on tetracycline bore tumors without X-gal
staining, demonstrating that tetracycline efficiently
repressed expression of the ICE-lacZ gene in vivo (Fig.
30 5B).
        By 4 days after removal of tetracycline, the
entire tumor, except for endothelial cells, expressed the
ICE-lacZ gene, as demonstrated by intense X-gal staining
(Fig. 5C). In contrast, in a comparable time point
35 animal maintained on tetracycline, repression of the

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ICE-*lacZ* gene was still clearly in effect, since no X-gal
staining was evident at this time (Fig. 5D). In animals
taken off tetracycline for 4 days, there were numerous
areas of cell death. The most intense region of cell
5 death was within the central part of the tumor with
smaller areas of cell death radiating out toward the
peripheral areas of the tumor. The neutral red
counterstain revealed areas of nuclear heterogeneity and
morphology suggestive of the nuclear condensation
10 associated with apoptotic cell death in a background of
X-gal positive staining (Fig. 5E). There were also, in
some areas, shrunken, rounded up cells that had detached
from the cellular architecture consistent with apoptosis
(Fig. 5F). Morphologic features of apoptosis were also
15 noted in tumors in animals 6 and 9 days after removal of
tetracycline (data not shown).

One animal, in which tetracycline was retained in
its drinking water for 9 days, demonstrated some X-gal
positive staining in the 9L-ICE-*lacZ* tumor; however, this
20 animal bore a large intracranial tumor and may have been
too lethargic to drink sufficient tetracycline water to
keep the gene off. *In situ* DNA end-labeling revealed
internucleosomal DNA fragmentation in condensed and
fragmented nuclei in areas of tumor cell death in animals
25 off tetracycline for 4 and 9 days (Fig. 5G), but not at
comparable time points in animals maintained on
tetracycline (data not shown).

D.   Effect of ICE-*lacZ* on Tumorigenicity
In parallel with the intracranial tumor study,
30 rats inoculated subcutaneously with 9L-ICE-*lacZ* cells
were kept on tetracycline for 7 days, then tetracycline
was removed from the drinking water of half of the
animals. Subsequent measurement of tumor volumes were
determined at two day intervals. The subcutaneous tumors

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in animals taken off tetracycline were statistically
smaller in size than the tumors in animals that were kept
on tetracycline (Fig. 5H).

IV.   Summary

5   IL-1B-converting enzyme (ICE) is a member of a
growing family of cysteine proteases shown to be a
crucial component in the activation of a genetic program
that leads to autonomous cell death in mammalian cells.
In this Example a murine ICE-lacZ fusion gene was
10 introduced into a novel retroviral vector designed to
achieve regulated ectopic expression of a foreign gene in
mammalian cells. By delivering the ICE-lacZ gene within
a retroviral vector and under the control of a
tetracycline regulated promoter, the intrinsic cell death
15 program of ICE was used as a means for tumoricidal
therapy in a rat brain tumor model. The results
demonstrated that both, in culture (in vitro) and in
vivo, suppression of ICE-lacZ expression was extremely
tight in the presence of tetracycline, as determined by
20 the lack of X-gal positive tumor cells and by cell
viability. When tetracycline was withdrawn, ICE-lacZ
gene expression was rapidly turned on and apoptosis -
mediated cell death (evidenced by widespread apoptosis
occurred as evidenced by tumor cell cytoplasmic
25 reduction, detachment of cells from the tumor
architecture, nuclear condensation, and internucleosomal
DNA fragmentation) occurred in essentially all tumor
cells.

V.   Potential Applications

30   Tumorigenesis may in part result from loss of a
cell's ability to undergo apoptosis in response to
physiologic stimuli (Hoffman, B. & Liebermann, D.A.
Molecular controls of apoptosis: differentiation/growth

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arrest primary response genes, proto-oncogenes, and tumor
suppressor genes as positiv & negative modulators.
Oncogene. 9, 1807-12 (1994)). By overexpressing a cell
death gene that encodes a protein likely responsible for
5 the final stages of the programmed cell death program,
this program can be initiated at a relative downstream
point and potentially bypass tumor cell resistance to
apoptosis. Indeed, the results described in this study
suggest this paradigm may be applicable in clinical
10 situations. In this regard, the tightly regulated
expression afforded by this retroviral vector *in vivo*
allows time for gene delivery e.g. grafting of packaging
cells into the tumor with infection of dividing cells
(Short, M.P., *et al.*Gene delivery to glioma cells in rat
15 brain by grafting of a retrovirus packaging cell line. *J
Neurosci Res.* 27, 427-39 (1990)) and then for precise
induction of death of both the tumor cells and packaging
cells. The packaging cells are thereby allowed to
migrate and infect tumor to increase gene transduction
20 efficiency and induced to undergo cell death along with
tumor to increase safety. This vector should be useful
in other gene therapy paradigms where precise induction
of gene expression is desirable. Both temporal and
quantitative control of the expression of a gene product
25 would augment the clinical applicability of gene therapy.
Furthermore, this vector could enhance the study of the
biochemical events during programmed cell death which
have been elusive due to the lack of a model exhibiting
synchronized progression into cell death.
30   Clearly there is an attraction to harnessing a
potent genetic program that is already in place in
vertebrates for tumoricidal therapy. Interestingly,
recent studies suggest that radiation therapy,
chemotherapy, and gene therapy (e.g. gangciclovir
35 activated by herpes simplex virus thymidine kinase),

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whos therapeutic effect had traditionally been
attributed to direct DNA damage, may exert their
cytotoxic effects through a programmed cell death
mechanism (Lowe, S.W., Schmitt, E.M., Smith, S.W.,
5 Osborne, B.A., & Jacks, T. p53 is required for
radiation-induced apoptosis in mouse thymocytes. *Nature*.
362 847-849 (1993); Lowe, S.W., Ruley, H.E., Jacks, T., &
Housman, D.E. p53-dependent apoptosis modulates the
cytotoxicity of anticancer agents. *Cell*. 74 957-67
10 (1993); and Freeman, S.M., *et al*. The "bystander effect":
tumor regression when a fraction of the tumor mass is
genetically modified. *Cancer Res*. 53, 5274-83 (1993)).

      As with many human gliomas, 9L gliosarcoma cells
contain a mutant form of p53 (Asai, A., *et al*. Negative
15 effects of wild-type p53 and c-Myc on cellular growth and
tumorigenicity of glioma cells. Implication of the tumor
suppressor genes for gene therapy. *J. Neuro-Oncol*. 19,
259-268 (1994); Louis, D.N. The p53 gene and protein in
human brain tumors. *J. Neuropath. and Exper. Neurol*. 53,
20 11-21 (1994)). It has recently been shown that cisplatin
induces ICE expression in murine and human glioma cell
lines during apoptosis regardless of their p53 status
(Kondo, S., Barna, B.P., Morimura, T., Takeuchi, J., &
Barnett, G.H. Cisplatin induces expression of
25 interleukin-1 beta converting enzyme in murine and human
glioma cells during apoptosis regardless of their p53
status. *Annual Meeting of the Congress of Neurological
Surgeons*. San Francisco, CA (1995)). Thus, induction of
apoptosis by the ICE-*lacZ* protein in 9L gliosarcoma cells
30 demonstrates that ICE function is downstream or
independent of p53 function and suggests that
ICE-mediated tumoricidal therapy is possible even in
tumors with mutant p53. As more cell death or growth
suppressive genes and their mechanism of action become
35 elucidated, these genes may be of use in the paradigm

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established in this study both as therapeutic tools and
as a means f studying the homeostasis of cell
proliferation and death.

EXAMPLE 3
Construction of a "Reverse" Drug
Regulatable Retroviral Vector

5   The drug regulated retroviral vector, as described
in Example 1 and Figure 6A, can be modified such that the
expression of a gene cloned into the response unit of the
10   retroviral vector is not expressed unless tetracycline is
present. Figure 6 compares the construct of the original
tetracycline regulated retroviral vector (Fig. 6A) with
the "reverse" tetracycline regulatable retroviral vector,
designated pBPSrTR-1 (see Figure 6B as compared to Figure
15   6A). The reverse tetracycline regulatable retroviral
vector is designed such that the tTA gene in pBPSTR-1
(see Figure 6B) has been removed, altered and then
replaced so that the altered tTA ["reverse" tTA (rtTA)]
requires the presence of tetracycline, tetracycline
20   derivatives or tetracycline analogues to bind promoter
elements within the response unit promoter and activate
the expression of the gene inserted into the response
unit. This was accomplished by first excising the tTA
gene from the pBSTR-1 retroviral vector with
25   HindIII/BamH1. Next, the "reverse" rtTA gene was
obtained from Dr. Bujard and the 5' and 3' ends were
modified using HindIII and BamH1 adapter primers and the
polymerase chain reaction (PCR) so that the adapted rtTA
gene could be inserted into pBPSTR-1, to construct the
30   retroviral vector pBPSrTR-1. In the version of the
tetracycline regulatable retroviral vector as described
in Example 1, expression of a gene inserted into the
multiple cloning site of the response unit is dependent
on tTA protein binding to tetO sequences in the response
35   unit. The tTA protein is different than the "reverse"

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tTA (rtTA) protein present in the "r verse" retroviral
vector (pBPSrTR-1) because it does not need to bind
tetracycline for it to also bind tetO sequences and
activate expression of a gene in the response unit.
5 However, in the original vector, when tetracycline does
bind tTA protein, this causes tTA to dissociated from
tetO sequences and turns off expression of a gene in the
response unit (See Figure 6A).

     In the reverse tetracycline regulatable retroviral
10 vector, expression of a gene inserted into the multiple
cloning site of the response unit is dependent on
"reverse" tTA (rtTA) protein binding to tetO sequences in
the response unit. The reverse tTA (rtTA) protein is
different than the tTA protein present in the original
15 retroviral vector (pBPSTR-1, Figure 6A) because it needs
to bind tetracycline for it to also bind tetO sequences
and activate expression of a gene in the response unit
(See Figure 6B).

     The applications for the "reverse" acting
20 tetracycline-regulatable retroviral vector will be the
same as those described for the original tetracycline
regulatable retroviral vector, pBPSTR-1.

                        EXAMPLE 4
        Treatment of Neurological Disorders Using a Rat Model
25   This Example demonstrates how a tetracycline
regulated retroviral vector can be used to treat
neurological disorders. Tyrosine hydroxylase (TH) is a
rate-limiting enzyme necessary for the biosynthesis of
dopamine and glial derived neurotrophic factor (GDNF) is
30 a neurotrophic factor important for motor and
dopaminergic neuron survival and uptake of dopamine in
dopaminergic neurons.

     Specifically, a retroviral vector containing
tetracycline inducible promoter elements was used in
35 these experiments which made it possible to deliver and

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r gulate the expression of a therapeutic gene in diseased
cells. In practice, expression of the TH and GDNF genes
are under the transcriptional control of a
tetracycline:VP16 transactivator fusion protein (tTA),
5 which positively regulates expression of the therapeutic
gene in the absence of tetracycline; however, in the
presence of tetracycline expression of the TH and GDNF
genes are repressed.
        A tetracycline regulatable retroviral vector
10 containing the GDNF gene and a tetracycline regulatable
retroviral vector containing the TH gene was constructed
as shown in Figure 7.
        Rat1 fibroblast cells were used in these
experiments because they serve as excellent models for
15 humans and can be used in a rat neurodegenerative model
that mimics quite well the human disorder. To
demonstrate the efficiency of retroviral delivery of the
TH and GDNF genes (Figure 7) and the tetracycline-
dependent gene expression of the delivered genes, Rat1
20 fibroblasts were infected with retrovirus containing
either the TH or GDNF gene using methods as described in
Example 2. Infected puromycin-resistant Rat1 fibroblasts
clones were examined for tetracycline dependent regulated
expression of the GDNF (Figure 8A, clones GD2, GD3, GD4,
25 GD5, GD6) and TH genes (see Figure 8B, clones TH1, TH3,
TH4). Shown in Figures 8A and 8B are western blots of
protein from puromycin resistant clones that had been
selected from pools of Rat1 cells that have been infected
with the tetracycline regulatable retrovirus. Clones
30 GD2, GD3, GD4, GD5, GD6, TH1, TH3, TH4 are different Rat1
clones selected with puromycin as described in the above
Examples. The "-" means that the clones were not treated
with tetracycline and "+" means that the clones were
treated with tetracycline). "C" is the non-infected Rat1
35 control cells. The results demonstrated that GDNF and TH

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gene expression was inhibited in tetracycline treated clones and GDNF and TH was expressed in clones not treated with tetracycline. These results demonstrate the gene delivery is very efficient and that expression of

5 the delivered genes can be tightly controlled with tetracycline.

Also, a rat model system will be used to determine whether restoring normal levels of dopamine to the brains of 6-hydroxydopamine treated rats using the tetracycline

10 regulated retroviral vectors of this invention can provide a curative therapy for this neurological disorder. The strategy to be used includes using rat fibroblasts (Rat1) clones that carry the TH and GDNF genes for implantation into the striatium of 6-

15 hydroxydopamine treated rats where they should manufacture the TH and GDNF proteins while remaining externally controlled via tetracycline. This approach is advantageous because it reduces the risk of rejection of the implant by the immune system and also adds the

20 tetracycline controlled expression component. Furthermore, the presence of GDNF should enable dopamine receptor-bearing cells to thrive and to more effectively uptake the new, higher level of dopamine resulting from TH activity.

25   EXAMPLE 5
              Treatment of Parkinson's Disease in Humans
          This Example demonstrates how a tetracycline regulated retroviral vector can be used to treat patients with Parkinson's disease.

30 I.   General Background on Parkinson's Disease
          Paralysis agitans, commonly known as Parkinson's disease, is a relatively common neurologic disorder of the brain where a specific class of dopaminergic neurons located in the nigrostriatal pathway deteriorate. The

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reason for this deg neration is unknown, although,
 xidative damage, envir nmental toxins, genetic
predisposition, and accelerated aging have all been
proposed to be possible factors in the disease.
5   Whichever the cause, the loss of these neurons can have a
profound impact on the afflicted individual. In part,
this is because dopaminergic neurons normally produce
dopamine and when they degenerate, dopamine levels in the
brain are substantially reduced. Dopamine is important
10 because it functions as a chemical messenger responsible
for transmitting signals between the substantia nigra and
the corpus striatum of the brain to produce smooth,
purposeful muscle activity. Loss or decreased levels of
dopamine causes the nerve cells of the corpus striatum to
15 fire 'out of control,' leaving patients unable to direct
or control their movements in a normal manner. Symptoms
of this disease include stiffness, tremor, slowness of
movement, difficulty with balance, and difficulty in
walking.
20   Drug therapy-based treatments for patients with
Parkinson's disease are currently in use to replace the
dopamine lost in the disease process. L-dopa (Levodopa;
an intermediate in the biosynthesis of dopamine) or
related agents, bring relief to many patients, but L-dopa
25 becomes ineffective over time and debilitating side-
effects develop with prolonged use. *Kupsch, A. & Oertel,*
W. H. (1994), Life Sciences, 55:2033-2095.
         A strategy involving intrastriatal implantation of
human fetal dopamine-rich mesencephalic tissue has shown
30 sustained improvement of motor function in Parkinsonian
patients. *Freed, C.R., Breeze, R.E., Rosenberg, N.L.*
Schneck, S.A., et al. (1992), New England Journal of
Medicine, 327:1549-1555. However, ethical issues
involving the use of abortive human fetuses as a source

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for dopamine-rich mesencephalic tissue has generated
considerable debate.

     An alternative to using fetal tissue as a source
for dopamine and neurotrophic factor production is to
5 genetically modify non-neuronal cells from the
Parkinsonian patient with the capacity to produce
dopamine and neurotrophic factors.

     This Example demonstrates a rat parkinson model
and teaches methods to genetically modify cells from the
10 Parkinsonian patient to increase and control the
production and uptake of dopamine.

     II.   Construction of a Tetracycline Regulated
             Retroviral Vector Containing the Gene Encoding
             Tyrosine Hydroxylase
15   A tetracycline regulated retroviral vector
containing the DNA encoding tyrosine hydroxylase (TH) was
constructed by employing the methods taught in Examples 1
and 2. Tyrosine hydroxylase is a rate-limiting enzyme
necessary for the biosynthesis of dopamine. Zigmond,
20 R.E., Schwarzschild, M.A. & Rittenberg, A.R. (1989),
Annu. Rev. Neurosci, 12:415-461. In this vector, the
human tyrosine hydroxylase (TH) cDNA was excised from the
plasmid pTH with BamH1 and cloned into the BamH1 site of
pBPSTR-1 placing the TH gene under the control of the
25 tetO/min CMV promoter in the response unit. Regulation
of this enzyme with or without the presence of
tetracycline, via employing a tetracycline regulated
retroviral vector, allows for the controlled expression
of this enzyme.

30   Although, other studies have used viral vectors to
deliver the TH gene to cells that potentially could
supply dopaminergic neurons with dopamine or L-dopa.
Gage, F.H, et al (1987), Neuroscience, 23:795-807; Gage,
F.H. & Fisher, L.J., et al (1991), Neuron, 6:1-12, this
35 approach is unique in that viral gene delivery vectors

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contains elements that allow for t tracycline regulated
expression f the delivered gene (pBPSTR-1) (Paulas, et
al., J. Virol gy in press).

    III.   Construction of a Tetracycline Regulated
5   Retroviral Vector Containing the Gene Encoding
            Glial-Derived Neurotrophic Factor
            A tetracycline regulated retroviral vector
containing the DNA encoding glial-derived neurotrophic
factor (GDNF) was constructed by employing the methods
10 taught in Examples 1 and 2. GDNF is a neurotrophic
factor important for motor and dopaminergic neuron
survival and uptake of dopamine in dopaminergic neurons.
The GDNF cDNA was excised from the plasmid pGDNF with
BamH1/Not1 and cloned into the BamH1/Not1 sites of
15 pBPSTR-1 placing the GDNF cDNA under the control of the
tetO/min CMV promoter in the response unit. Lindsay,
R.M. (1995), Nature, 373, 289. Regulation of GDNF with
or without the addition of tetracycline, via employing a
tetracycline regulated retroviral vector, allows for the
20 controlled expression of this enzyme.

    IV.   Isolate and Infect Fibroblasts from Patients with
            Parkinson's Disease
            Based on studies using rat fibroblasts isolated
from rat brain and then reimplanted back into a rat
25 parkinson model brain, brain-derived fibroblast cells are
isolated from patients with Parkinson's disease. These
cells will be infected with the tetracycline regulated
retroviral vector containing the TH or GDNF gene as
described in Sections II and III above and reimplanted
30 back into the brain of the Parkinson patient.
            Because of the design of these vectors, tyrosine
hydroxylase or GDNF will be synthesized constitutively
until tetracycline is administered to the patient. Thus,
tetracycline can be used to control the production of

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tyrosine hydroxylase and GDNF in a patient with
Parkins n's disease.

Thus, the tetracycline regulated retroviral
vectors of this invention not only provide a means of
5 regulating expression of a gene, e.g., TH or GDNF, in an
"OFF" or "ON" manner, but also allow for controlling
intermediate levels of expression. This could be
important during periods when more or less amounts of the
TH or GDNF are required by the Parkinson patient. The
10 level of GDNF or TH expressed can be achieved by
increasing or decreasing the pharmacological levels of
tetracycline; this allows for controlling situations
where greater or less dopamine is needed.

Rat1 fibroblast cells have been used in similar
15 experiments (See Example above), and one of ordinary
skill in the art knows that the rat can serve as an
excellent model for humans. Thus, success in the rat
model provides evidence that the same techniques using
human compatible promoters, etc, will be effective in
20 treating patients with Parkinson's disease.

Other embodiments are within the following claims.

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1. A tetracycline regulated retroviral vector capable of deliv ring and controlling the expression of a heterologous g ne in mammalian cells, comprising the following DNA sequences operatively linked from 5' to 3':

    (a)   DNA comprising a first promoter;

    (b)   DNA comprising a second promoter different from the first promoter in (a) and different from the third promoter in (e);

    (c)   DNA encoding a tetracycline regulator unit (tTA) under the transcriptional control of said second promoter;

    (d)   DNA encoding a tetracycline response unit (tetO) in an antisense orientation relative to said DNA encoding said regulator unit of (c); and

    (e)   DNA comprising a third promoter different from the first promoter in (a) and different from the second promoter in (b) wherein said DNA encoding said response unit of (d) is under the transcriptional control of said third promoter.

2. The vector of claim 1 wherein the first promoter of (a) is specific for the cell type that said vector infects.

3. The vector of claim 1 wherein the first promoter of (a) is specific for the tissue type that said vector infects.

4. The vector of claim 1 wherein said first promoter of (a) is selected from the group consisting of 5' retroviral long terminal repeat promoter, Rous Sarcoma Virus (RSV), Human Immunodeficiency Virus (HIV), and phosphoglycerate kinase.

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5.   The vector f claim 1 further containing DNA
encoding a mammalian protein selected from the group
consisting of ICE, TH, GDNF, and TH, and wherein said DNA
is under the transcriptional control of said first
5 promoter of (a).

6.   The vector of claim 5 wherein said protein is
heterologous to the cells that said vector infects.

7.   The vector of claim 5 wherein the addition of
tetracycline increases or enhances the expression of said
10 mammalian gene.

8.   The vector of claim 5 wherein the addition of
tetracycline inhibits or suppresses the expression of
said mammalian gene.

9.   The vector of claim 1 wherein said second
15 promoter of (b) is selected from the group consisting of
SV40 promoter, JC virus promoter, glial fibrillary,
nestin, P0, estrogen receptor, and phosphoglycerate
kinase.

10.   The vector of claim 1 wherein said third
20 promoter of (e) is selected from the group consisting of
CMV promoter and thymidine kinase.

11.   The vector of claim 1, further comprising an
endonuclease site located in an antisense orientation
relative to said DNA encoding said regulator unit of (c).

25   12.   The vector of claim 11 wherein said
endonuclease site is selected from the group consisting
of Xho1, Spe1, Sfi1, and Bstx1.

13. The vector of claim 1 further comprising a
DNA encoding a selectable marker, under the
transcriptional control of said first promoter in (a).

14. The vector of claim 13 wherein said
5 selectable marker is selected from the group consisting
of puromycin, neomycin, hygromycin and thymidine kinase.

15. A tetracycline regulated retroviral vector
capable of delivering and controlling the expression of a
heterologous gene in mammalian cells, comprising the
10 following DNA sequences operatively linked from 5' to 3':

- (a) DNA comprising a first promoter;
- (b) DNA encoding a selectable marker under the
       transcriptional control of said first
       promoter in (a);
- 15 (c) DNA comprising a second promoter different
       from the first promoter in (a) and different
       from the third promoter in (g);
- (d) DNA encoding a tetracycline regulator unit
       (tTA) under the transcriptional control of
- 20   said second promoter;
- (e) an endonuclease site located in an antisense
       orientation relative to said DNA encoding
       said regulator unit of (d);
- (f) DNA encoding a tetracycline response unit
- 25   (tetO) in an antisense orientation relative
       to said DNA encoding said regulator unit of
       (d);   and
- (g) DNA comprising a third promoter different
       from first promoter in (a) and different from
- 30   second promoter in (c) wherein said DNA
       encoding said response unit of (f) is under
       the transcriptional control of said third
       promoter.

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16. The vector of claim 15 wherein the first
promoter of (a) is specific for the cell type that said
vector infects.

17. The vector of claim 15 wherein the first
5 promoter of (a) is specific for the tissue type that said
vector infects.

18. The vector of claim 15 wherein said first
promoter of (a) is selected from the group consisting of
5' retroviral long terminal repeat promoter, Rous Sarcoma
10 Virus, phosphoglycerate kinase and Human Immunodeficiency
Virus.

19. The vector of claim 15 further containing DNA
encoding a mammalian protein selected from the group
consisting of ICE, TH, GDNF, and TH, and wherein said DNA
15 is under the transcriptional control of said first
promoter of (a).

20. The vector of claim 19 wherein said gene is
heterologous to the cells that said vector will infect.

21. The vector of claim 19 wherein the addition
20 of tetracycline increases or enhances the expression of
said mammalian gene.

22. The vector of claim 19 wherein the addition
of tetracycline inhibits or suppresses the expression of
said mammalian gene.

25   23. The vector of claim 15 wherein said second
promoter of (c) is selected from the group consisting of
SV40 promoter, JC virus promoter, glial fibrillary acidic

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protein, nestin, P0, estrogen receptor, and
phosphoglycerate kinase.

24. The vector of claim 15 wherein said third
promoter of (g) is selected from the group consisting of
5   CMV promoter, and thymidine kinase.

25. The vector of claim 15 wherein said
endonuclease site of (e) is selected from the group
consisting of Xho1, Spe1, Sfi1, and Bstx1.

26. The vector of claim 15 wherein said
10 selectable marker of (b) is selected from the group
consisting of puromycin, hygromycin, neomycin and
thymidine kinase.

27. The vector of claim 15 wherein said first
promoter is the 5' retroviral long terminal repeat
15 promoter, said selectable marker is puromycin resistance
gene, said second promoter of (c) is selected from the
group consisting of SV40 promoter and JC virus promoter,
, and wherein said third promoter of (g) is CMV promoter.

28. A method of inducing apoptosis in a cell,
20 comprising the steps of:
        (a) providing a tetracycline regulated retroviral
             vector containing DNA encoding the IL-1 Beta-
             converting enzyme (ICE), or functional
             homologues of ICE, under the control of
25   tetracycline; and
        (b) introducing said vector into said cell.

29. The method of claim 28 wherein the presence
of tetracycline inhibits or suppresses expression of said
ICE encoding DNA.

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30. The method of claim 28 wherein the presence
of tetracycline enhances or increases expression of said
ICE encoding DNA.

31. A method of delivering a mammalian
5 regulatable or inducible gene to a human patient
comprising the steps of:
   (a) obtaining cells for implantation;
   (b) introducing into said cells of (a) a
        tetracycline regulated retroviral vector
10   containing said mammalian gene, wherein said
        gene is under the control of tetracycline;
        and
   (c) implanting said cells of (b) into said
        patient.

15   32. The method of claim 31 wherein said cells of
(a) are obtained from said patient.

33. The method of claim 31 wherein said
tetracycline in (b) inhibits or suppresses the expression
of said gene.

20   34. The method of claim 31 wherein said gene of
(b) is selected from the group consisting of ICE,
tyrosine hydroxylase, and glial derived neurotropic
factor.

35. The method of claim 31 wherein tetracycline
25 enhances or increases the expression of said gene.

36. A method of delivering a regulatable or
inducible mammalian gene to a mammal comprising the steps
of:

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    (a)   obtaining viral particles containing
tetracyclin regulated retroviral vectors
containing said mammalian gene; and

    (b)   infecting cells by administering said viral
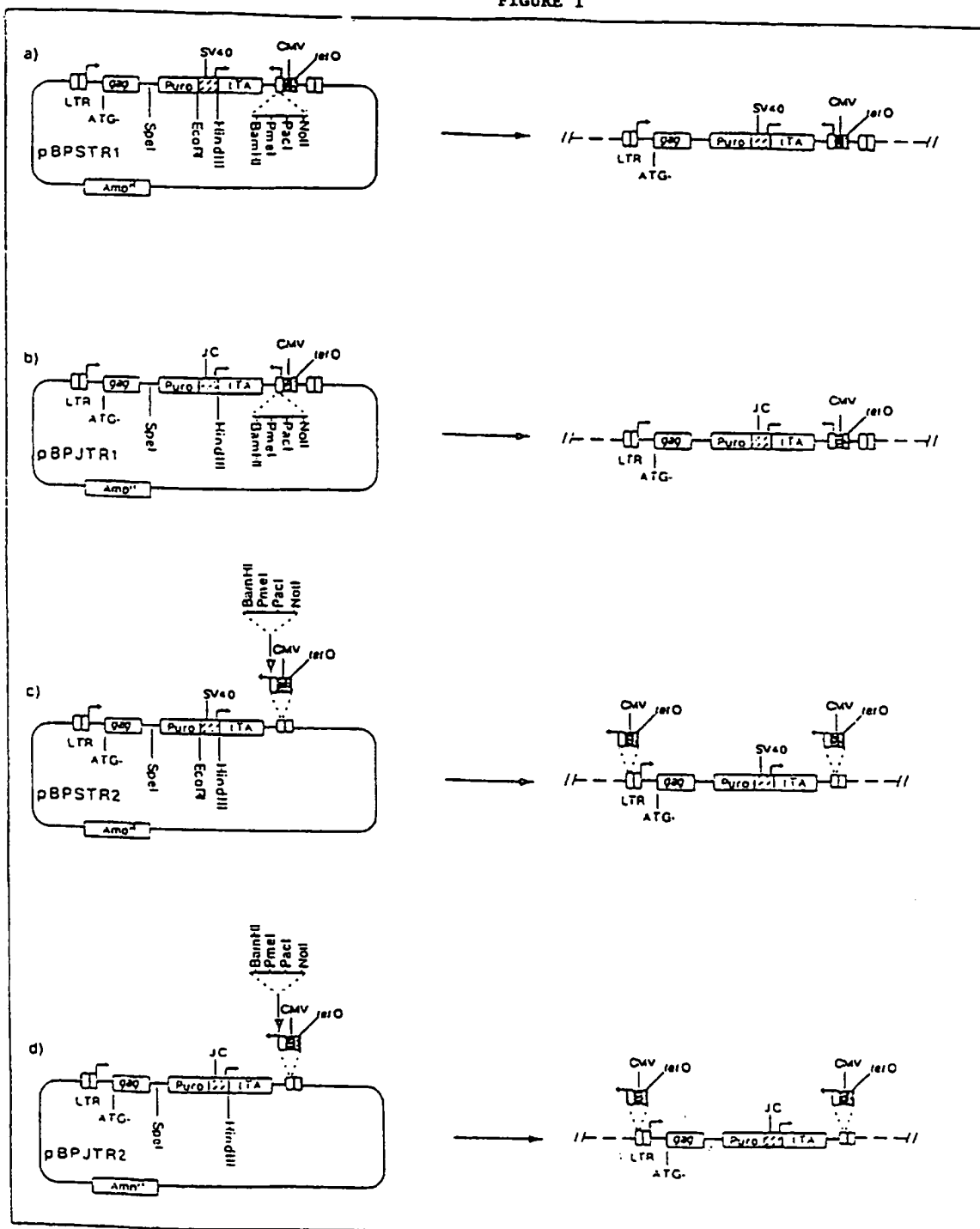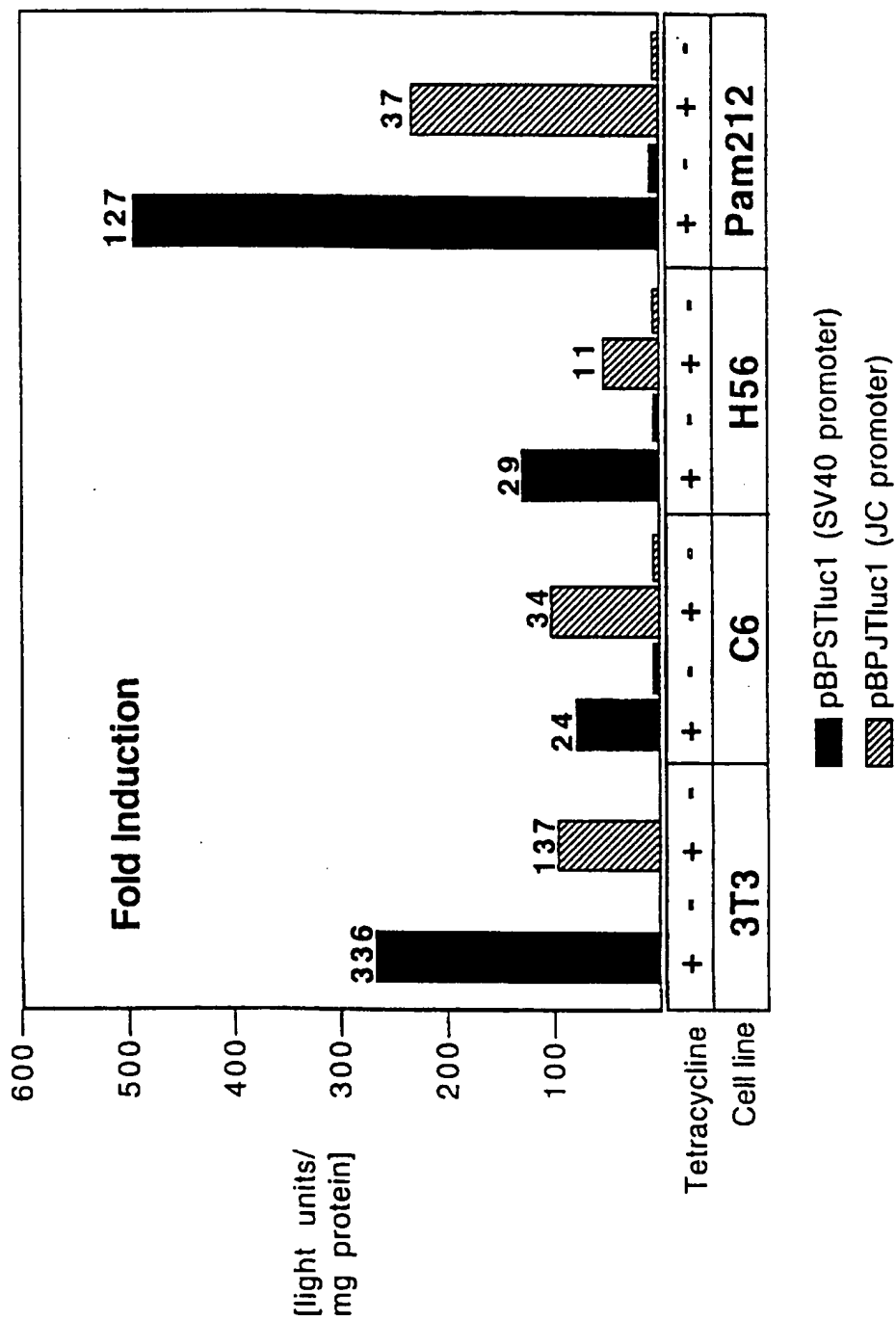5   particles of (a) to an affected organ of said
mammal.

37.   The method of claim 36 wherein said mammalian
gene of (a) is selected from the group consisting of ICE,
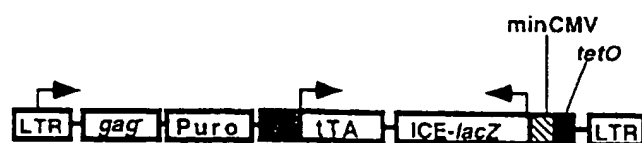tyrosine hydroxylase, and glial-derived neurotropic
10 factor.

FIGURE 1

FIGURE 2

Regulation of Retroviral Vectors in Different Cell Types

Fold Induction

[light units/ mg protein]

| Tetracycline | + | - | + | - | + | - | + | - | + | - | + | - | + | - |
| Cell line | 3T3 | | | C6 | | | H56 | | | Pam212 | | |

■ pBPSTluc1 (SV40 promoter)

▨ pBPJTluc1 (JC promoter)

FIGURE 3

A

B
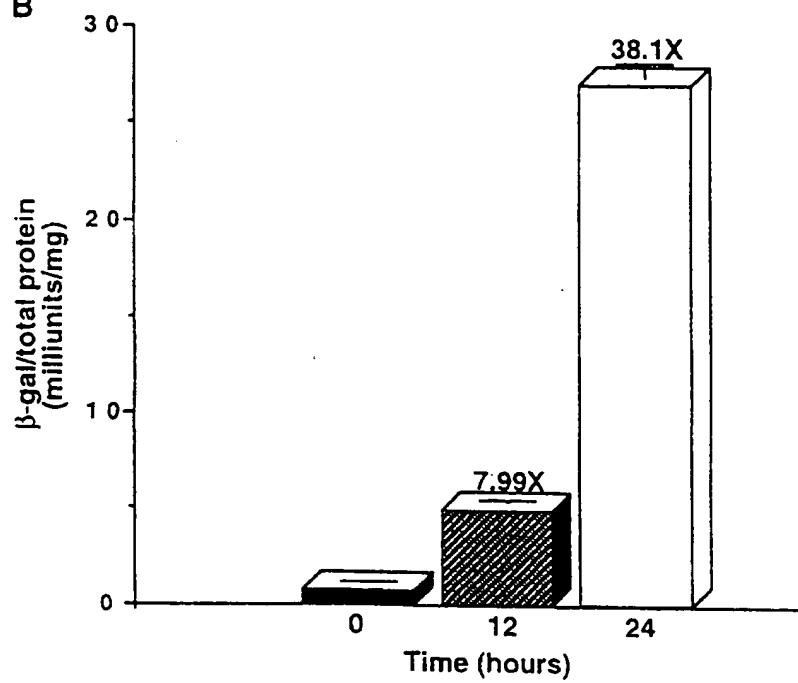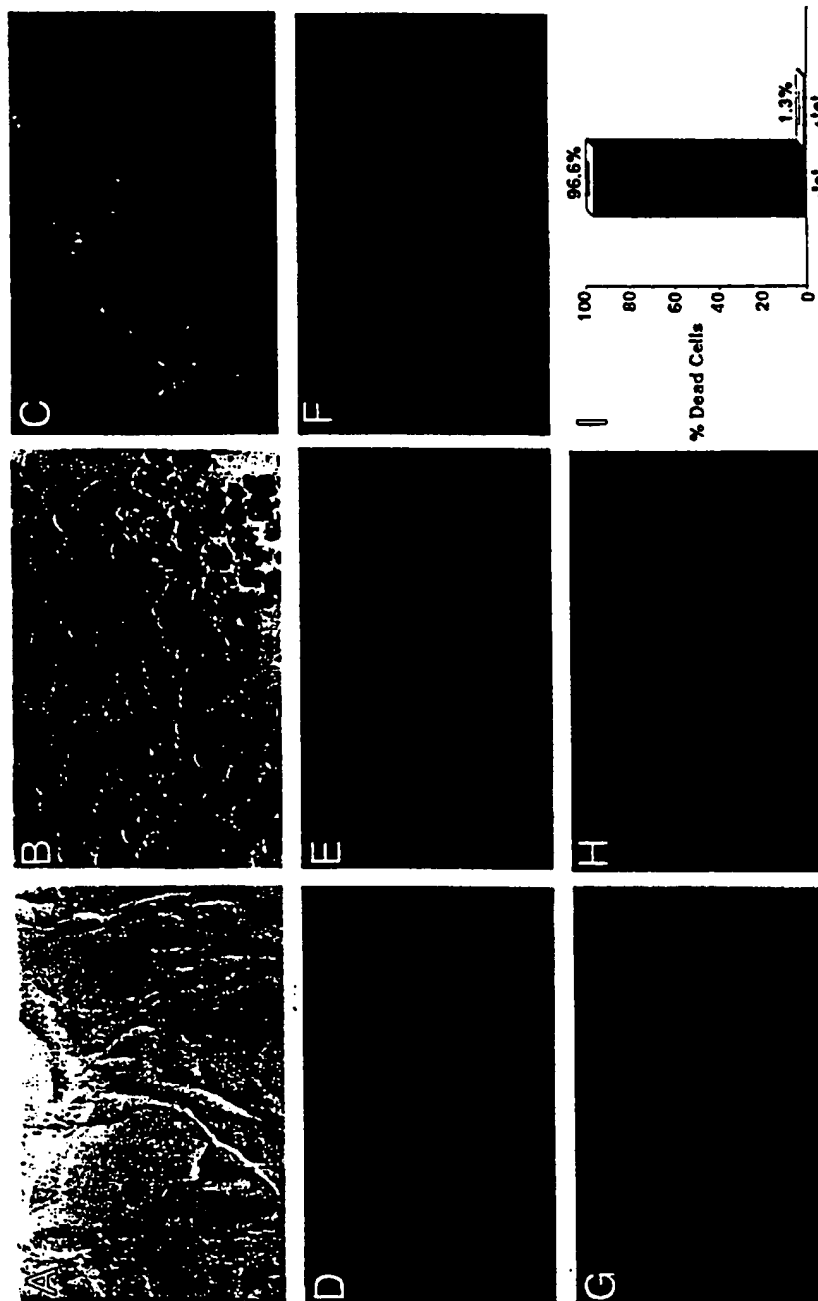
FIGURE 4

Figure 5
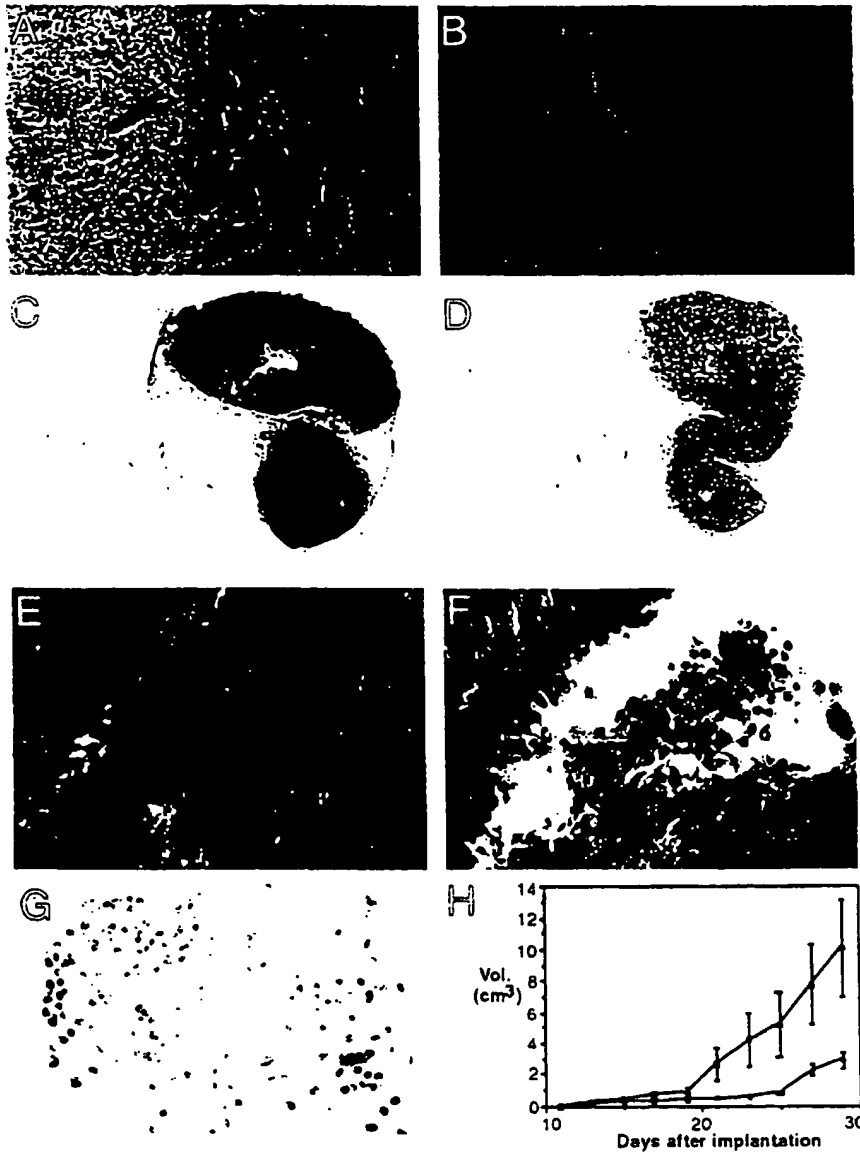
6/8

FIGURE 6

A

Tetracycline   regulatable   retroviral   vector
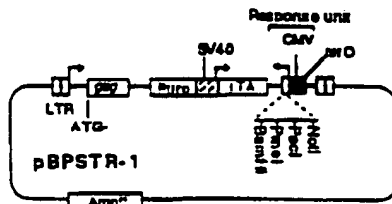
In this original version of the tetracycline-regulatable retroviral vector, expression of a gene inserted into the multiple cloning site of the response unit is dependent on tTA protein binding to tetO sequences in the response unit. The tTA protein is different than the reverse tTA (rtTA) protein present in the retroviral vector (pBPSrTR-1) because it does not need to bind tetracycline for it to also bind tetO sequences and activate expression of a gene in the response unit. However, when tetracycline does bind tTA protein, this causes tTA to dissociated from tetO sequences and turn off expression of a gene in the response unit.

B

Reverse   tetracycline   regulatable   retroviral   vector
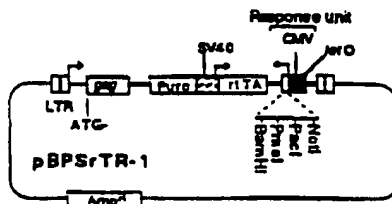
In this version of the tetracycline-regulatable retroviral vector, expression of a gene inserted into the multiple cloning site of the response unit is dependent on reverse tTA (rtTA) protein binding to tetO sequences in the response unit. The reverse tTA (rtTA) protein is different then the tTA protein present in our original retroviral vector (pBPSTR-1) because it needs to bind tetracycline for it to also bind tetO sequences and activate expression of a gene in the response unit.
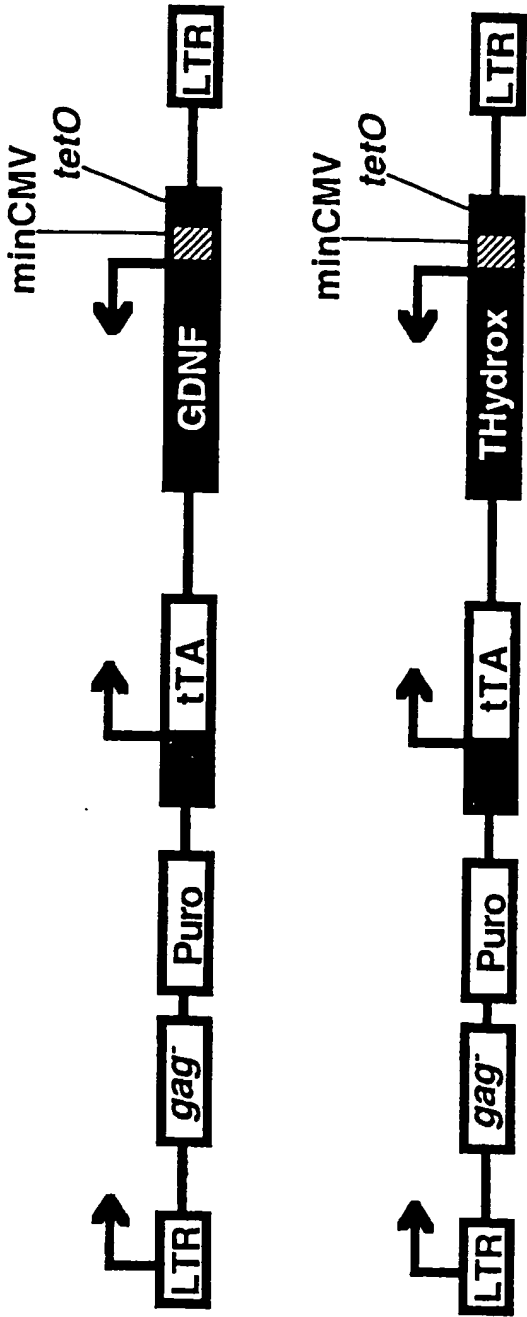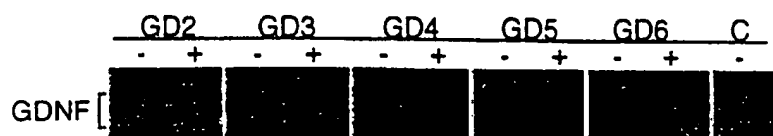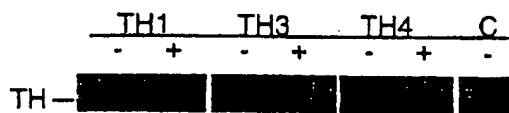
FIGURE 7

FIGURE 8

A

Western blot of clonal Rat1 cells probed with GDNF antibody

B

Western blot of clonal Rat1 cells probed with TH antibody

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/U⬤873

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A01N 43/04; A61K 31/70; C12N 15/09, 15/10
US CL :514/44; 424/93.21; 435/320.1, 69.1, 172.3, 240.2; 536, 23.4, 24.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 424/93.21; 435/320.1, 69.1, 172.3, 240.2; 536, 23.4, 24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, CAPLUS, WPIDS
search terms: regulatable, retrovirus, tetracycline, ICE, IL-1B-converting enzyme, multiple promoters

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,464,758 A (GOSSEN et al.) 07 November 1995, column 2, lines 14-67 and column 3, lines 1-20.	1-37
Y	O'MALLEY, JR. et al. Somatic Gene Therapy. Archives of Otolaryngology-Head & Neck Surgery. October 1993, Vol.119, pages 1100-1107, especially pages 1104-1105.	28-37
Y	FISHMAN et al. Tetracycline-Regulated Cardiac Gene Expression In Vivo. Journal of Clinical Investigation. April 1994, Vol. 93, pages 1864-1868, especially pages 1867-1868.	1-37
Y	CERRETTI et al. Molecular Cloning of the Interleukin-1β Converting-Enzyme. Science. 03 April 1992, Vol. 256, pages 97-100, see entire document.	5, 19, 28, and 37

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search	Date of mailing of the international search report
01 FEBRUARY 1997	0 7 MAR 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	JILL SCHMUCK
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 89/03882 A1 (IMMUNEX CORPORATION) 05 May 1989, see entire document.	1-27